Fall 2020

APPLYING ENVIRONMENTAL SURVEILLANCE AND EPIDEMIOLOGY TO MANAGE Vibrio parahaemolyticus RISKS ASSOCIATED WITH SHELLFISH CONSUMPTION

Christopher Andrew Schillaci
University of New Hampshire, Durham

APPLYING ENVIRONMENTAL SURVEILLANCE AND EPIDEMIOLOGICAL REPORTING TO MANAGE *Vibrio parahaemolyticus* RISKS ASSOCIATED WITH SHELLFISH CONSUMPTION

BY

CHRISTOPHER SCHILLACI

B.A., UNIVERSITY OF WISCONSIN- MADISON, 2009

THESIS

Submitted to the University of New Hampshire
In Partial Fulfillment of
The Requirements for the Degree of

Master of Science
in
Natural Resources:
Environmental Conservation and Sustainability

September, 2020

This thesis has been examined and approved in partial fulfillment of the requirements for the degree of Masters of Science in Natural Resource Management by:

Thesis Director, Stephen H. Jones
Research Associate Professor of Natural Resources and the Environment, University of New Hampshire

Cheryl A. Whistler
Professor of Molecular, Cellular, and Biomedical Sciences, University of New Hampshire

Kenneth La Valley
Vice Provost, University Outreach & Engagement, University of New Hampshire

Angelo DePaola
Retired Lead Seafood Microbiologist, US Food and Drug Administration

On, August 14, 2020

Original approved signatures are on file with the University of New Hampshire Graduate School.

ACKNOWLDEGMENTS

I would like to thank the following individuals for helping me with this endeavor:

Dr. Steve Jones: Steve, Thank you for all of the advice, and the time you have dedicated to helping me grow as a biologist, student, and friend. This would not have been possible without your guidance and support.

Diane Regan: Diane, Thank you for your mentorship and support academically, professionally, and personally. Without your tireless work in the laboratory none of this would have been possible.

Dr. Cheryl Whistler: Cheryl, your work will undoubtable change the future of environmental surveillance and epidemiology. Your efforts to characterize the emergence of pathogenic strains in the region provided the framework for many of my most meaningful conclusions.

Dr. Andy DePaola: Your contributions to the field are legendary. I do not believe that there is anyone who has done more to advance the understanding of Vp. risk dynamics in the U.S. than you. Your willingness to come to Massachusetts and work closely with managers, industry, and researchers made a significant difference in the trajectory of infection occurrence in the state.

My Wife: For always believing in me and giving me the encouragement I needed to follow through with this. Thank you for not letting me give up and for being my number one fan.

Funding Sources: ISSC
NOAA Sea Grant Program

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER I

CHAPTER II

CHAPTER III

LIST OF FIGURES

CHAPTER II PAGE

Chapter III

ABSTRACT

APPLYING ENVIRONMENTAL SURVEILLANCE AND EPIDEMIOLOGY TO MANAGE *Vibrio parahaemolyticus* RISKS ASSOCIATED WITH SHELLFISH CONSUMPTION

by

Christopher Schillaci

University of New Hampshire, September 2020

Vibrio parahaemolyticus (Vp.) outbreaks and sporadic cases associated with the consumption of oysters harvested from the New England Region of the U.S. have increased since 2011. Increasing Vp. infections in Massachusetts have resulted in harvest areas closures, product recalls, and the implementation of costly control measures on the harvesting and handling of oysters in the state during summer months. A combination of factors such as increased summertime production and consumption of raw shellfish, and climate related changes leading to warmer sea surface temperatures and more variable salinities favorable for Vp. bacteria, have been attributed to the recent increase in infections. However, the 2012 introduction and ecosystem establishment of a highly virulent Pacific-endemic strain of Vp. called sequence type (ST) 36 has been implicated in most illnesses and appears to be driving risk. The growing public health and economic burden associated with managing Vp. risk in Massachusetts have made the collection of information to support the development of effective Vp. mitigation strategies and risk assessment models a major priority in the region.

Northeast oyster production is targeted almost exclusively toward the raw half shell market and Massachusetts production has increased nearly fivefold between 2008 and 2018, from 11 million to over 50 million oysters. Thus, the observed reported increase in Vp. illnesses

in the state may not reflect a significant change in the risk per serving to consumers. Both production and illnesses vary across harvest areas and should be evaluated to accurately assess effects of environmental conditions on exposure and risk. Massachusetts collected limited information on the distribution and abundance of total and potentially pathogenic (tdh+ and trh+) Vp. in shellfish harvest areas prior to the introduction of ST36 to inform Vp risk assessment models and localized risk management strategies. Our aim for this study was to characterize trends in Vp. infection risk in Massachusetts harvest areas in relation to environmental conditions, total and potentially pathogenic Vp. abundance, and clinical strains implicated in regional infections. Characterizing Vp risk in MA is critical to managing risk and sustaining the oyster industry.

We conclude:

- There is significant variability in Vp. risk between Massachusetts harvest areas
- Current Vp. surveillance methodology and temperature driven risk assessment models cannot accurately capture differential risk
- Spatial variability in Vp. population composition is likely the largest driver of differential Vp. infection risk between Massachusetts harvest areas.

CHAPTER I

INTRODUCTION

Molluscan shellfish are filter feeders and therefore have the ability to concentrate microorganisms and contaminants in their tissues at levels >100 times higher than observed in surrounding waters (1- 2). As a result of this filter feeding behavior, it is possible for shellfish consumers to be exposed to infectious levels of pathogens when consuming shellfish harvested from areas contaminated with human pollutants or that harbor naturally occurring human pathogens (2-4). Additionally, many bacterial pathogens can reproduce in shellfish if they are exposed to warm temperatures following harvest and during transportation and storage, further increasing the levels of pathogens in shellfish (6).

In 1984, the U.S. Food and Drug Administration (FDA) and the Interstate Shellfish Sanitation Conference (ISSC) developed a formal cooperative program known as the National Shellfish Sanitation Program (NSSP) for the regulation of shellfish in inter-state commerce in the U.S. The NSSP's Model Ordinance (MO) outlines requirements associated with the harvest area classification, harvest practices, processing, labeling, storage, handling, packing, shipping, harvester and dealer permitting, and illness risk mitigation and response; and ensures shellfish sanitation through the cooperation of state and federal control agencies, the shellfish industry, and the academic community. State Shellfish Control Authorities (SCAs), generally administered by public health and marine resource agencies, are responsible for administering these standards in their respective states. In many cases the MO requirements are developed in a way that allows SCAs to meet NSSP standards through the development of state specific controls that best apply to their industry and harvest areas, but ensure baseline protection (7).

In the MO there are several recognized naturally occurring pathogens that must be addressed by SCAs through an annual risk assessment or contingency plan. If it is determined that the risk of shellfish derived illness from a particular pathogen is likely to occur, SCAs are required to put in place control measures and ensure the ability to respond if they are notified of an outbreak associated with shellfish consumption. These controls may include consumer advisories, the implementation of harvest and/or handling requirements, restrictions on harvest based on environmental conditions that favor pathogen growth, closures when outbreaks are reported, and in the case of naturally occurring pathogens where regulatory thresholds have been established, proactive harvest area closures informed by environmental surveillance. In the case of naturally occurring pathogens where no regulatory threshold or reliable methods to monitor for pathogens in the environment exists, managers often must rely on an overly conservative or reactive approach to illness prevention and response. Such is the case for a number of species of bacteria in the genus *Vibrio* that are commonly associated with shellfish derived human illness in the U.S. (7)

Vibrio *Spp.*

Vibrio bacteria are ubiquitous in coastal and marine waters, and are a major constituent of the bacterial community of marine and estuarine ecosystems (3, 14-16). Vibrios can colonize marine plant and animal species and can be found on raw seafood products on the market (9, 10). Twelve species of *Vibrio* are reported to cause illness in humans (8). Illness from *Vibrio* spp. is collectively called vibriosis. In the U.S. vibriosis is a reportable disease through the Center for Disease Control (CDC) Cholera and other Vibrio Information System (COVIS) (11).

Vibrio parahaemolyticus

Vibrio parahaemolyticus (Vp.) is responsible for the majority of cases of vibriosis in the U.S. and is the leading cause of seafood related bacterial food borne illness worldwide (12-13). Gastric infection from pathogenic strains of Vp. can cause self-limiting gastroenteritis and in rare cases septicemia (14, 17). The severity of infection is strongly associated with underlying medical conditions (1), however, most infections are resolved without medical treatment (11). The human infective dose for Vp. is not known, and can vary with strain type and host susceptibility (18). Feeding studies conducted in the 1970's identified an infectious dose between 10^5 to 10^8 organisms per gram of oyster tissue (19-20). The USFDA has established guidance suggesting a < 10,000 MPN/g (4 log10 MPN/g) threshold for total Vp. in shellfish represents a low risk of infections (1), but currently in the U.S there are no regulatory thresholds for Vp. in shellfish meats or harvest area waters (7). Thoroughly cooking seafood products generally removes the risk of gastric Vp. infection, and most seafood borne cases of gastric Vp. infections are associated with the consumption of raw or undercooked shellfish. Raw oysters and clams are the number one reported vector for human gastric infections of Vp. due to their ability to concentrate Vp. in the digestive tissues, and consumer trends favoring raw consumption (3-6).

In 1999, the CDC estimated the total annual incidence of Vp. illness in the U.S. was 7,880 illnesses, and of that 65% were estimated to be food related (1). This estimate was based on an under-reporting factor of 1 reported case for every 20 cases that are not reported (21). In 2011, CDC updated its underreporting factor for Vp. to 1.1 reported cases for every 142.4 unreported cases; and CDC currently estimates that there are between 45,000-65,000 seafood-borne Vp. infections annually in the U.S., with approximately 80% associated with raw oyster and clam consumption (13, 22, Table 1). With increasing access to health care in the U.S.,

increasing state reporting requirements for enteric diseases, and the development of rapid culture independent diagnostic methods, many question the accuracy of the current under-reporting factor used by the U.S. CDC for Vp. (23).

Year	Vp. cases with seafood vector information available	Percent of Vp. cases reported associated with oyster consumption	Percent of Vp. cases reported to be associated with clam consumption	Estimated Cases Associated with Shellfish Consumption (1.1/142.4 under-reporting factor)
2007	180	60%	20%	20,505
2008	234	62%	14%	25,324
2009	329	57%	22%	37,011
2010	338	62%	21%	39,948
2011	262	54%	19%	27,235
2012	376	60%	21%	43,369
2013	520	71%	21%	68,124
2014	537	70%	15%	64,998
2015	544	68%	12%	61,972
Total	3,320	63%	18%	38,2942

Table 1. Seafood related *Vp.* cases by year 2007-2015 reported to CDC and shellfish species consumed *Cases may have consumed more than one type of seafood. From Burdette, 2017

Between 2007 and 2015 the number of reported foodborne Vp. cases in the U.S. more than tripled (Table 1), making Vp. one of the few foodborne bacterial pathogens reported to be on the rise in the U.S. (11). While this trend is concerning, it is important to understand how epidemiological data relative to Vp. illness occurrence is collected and potential limitations and bias in the data. For example, the CDC reported that in 2015 approximately 550 cases of seafood borne Vp. infections were reported to state health officials. Overall, this represents a 260% increase as compared to 2007 (Figure 1). These numbers, however, may not reflect actual illness trends relative to exposure or the "risk per serving." When considered in light of reported major increases in U.S. cultured oyster production, which are primarily consumed raw and shifting

seasonality of raw consumption to summer months, reported illnesses relative to servings may not have changed, or may actually be decreasing. Additionally, there are significant challenges preventing Vp. illness source attribution. Between 2007 and 2015 less than 30% of the shellfish related Vp. infections reported to CDC had associated trace-back information, and only 20% identified a likely harvest area (22). The lack of source attribution data associated with reported illnesses severely limits the ability for managers to apply epidemiological reporting to evaluate the Vp. risk per serving in shellfish harvest areas. Additionally, the lack of source attribution information impacts the ability for SCAs to implement harvest area closures and product recalls in response to reported Vp. illnesses in a manner that results in meaningful consumer protection. Further, COVIS data pertaining to Vp. infections is categorized based on the reporting location, not the presumptive source location. As improved cold chain and transportation economics have opened national and global markets for fresh U.S. seafood, reporting location provides little insight into source location (24).

Increasing* Vp. *Case Occurrence in the Northeast U.S.

Despite challenges with source attribution, over the last decade documented increases in Vp. infections have been associated with locally harvested oysters from the Northeast U.S. This increase has led to costly harvest area closures, product recalls and the implementation of stringent harvest and handling controls during periods when Vp. cases have occurred in the region (26). Several factors have been attributed to the reported increase in Northeast Vp. infections, including improved illness surveillance, increased oyster production targeted at the raw half-shell market, climate change extending the seasonal and geographical range of endemic pathogenic strains, and the human meditated introduction of a highly virulent non-resident Vp. lineage (25-30). For example in 2012 and 2013, a highly virulent pathogenic lineage of Vp.

endemic to the Pacific resulted in the largest oyster-associated Vp. outbreak on the Atlantic coast, tripling the 2012 and 2013 Atlantic Vp. case mean relative to the previous 5-year period (28-30). While previous U.S. outbreaks have been attributed to long-distance dispersals of non-native Vp. strains, in most cases these strains have rarely persisted to cause recurrent infections in subsequent years (26). However, ST36 has seemingly established environmental reservoirs in some New England harvest areas as it continued to be implicated in the majority of Massachusetts infections between 2014 and 2017 (28, 31). Additionally, a resident strain (ST631) has also caused infections from Massachusetts sources and remains the second most prevalent strain from clinical sources in the state (28- 29, 31).

Vp. Risk Management

Due to the lack of reliable microbiological standards for Vp. that can inform proactive management, management strategies in the U.S. primarily consist of risk mitigation through the development of control measures intended to reduce post-harvest growth of Vp. in shellfish, and illness reporting and response requirements (7, 18). The ISSC has established requirements in the NSSP MO for SCAs related to the assessment and mitigation of Vp. risk associated with the consumption of raw molluscan shellfish. In order to determine if the implementation of control measures to reduce the probability of Vp. illness occurrence are warranted, SCAs are required to annually conduct a risk assessment to determine if the risk of Vp. infection from the consumption of oysters is reasonably likely to occur in areas under their jurisdiction. In areas where environmental conditions or historic Vp. illness occurrence demonstrate that the risk of Vp. infection may be present, the NSSP requires State Shellfish Control Authorities to implement a Vp. control plan (VCP). The goal of the VCP is to reduce the probability of occurrence of Vp. illness through the establishment of time temperature controls or other

measures, such as post-harvest processing or proactive closures, during periods that have been historically associated with annual illnesses (7, 18).

The FDA published a quantitative risk assessment to characterize the factors influencing the public health impact associated with the consumption of raw oysters containing pathogenic Vp. The assessment used background information on observed Vp. abundance in a number of harvest areas across the U.S. and species-specific growth curves to estimate the levels of the Vp. in oysters through post-harvest handling, processing, and storage. Using the data from feeding studies and epidemiological investigations a dose-response model was developed to estimate the risk of Vp. per serving of oysters from six regions in the U.S. and test "what-if" scenarios to evaluate the likely impact of potential control strategies to reduce exposure to pathogenic Vp. from the consumption of raw oysters. The assessment largely relied on the general relationship between increasing water and air temperatures resulting in increased Vp. abundance and generalized risk by season and region. However, the authors, admittedly, did not account differences in pathogenic strain virulence or growth rates that have been increasingly recognized as an important factor in Vp. infection risk (1).

The primary risk mitigation strategy currently employed in the U.S. involves the rapid cooling of shellfish intended to prevent post-harvest growth of Vp. in shellfish. This approach was largely informed by the FDA Vp. risk assessment; and is consistent with regulatory requirements for other seafood products (1). A number of studies have shown rapid cooling of shellfish on direct ice, ice/water mixtures, and mechanical refrigeration to an internal temperature of 10°C can significantly reduce post-harvest growth of Vp. in shellfish at harvest, (32- 33), and a number of states have seen reductions in Vp. illness occurrence since the implementation of rapid cooling control measures (31, 34).

Environmental Vp. Surveillance

While rapid cooling and maintaining product under refrigeration following harvest until consumption can slow or stop Vp. growth in harvested shellfish (1, 32, 33), it does not eliminate the infection risk associated with the consumption of shellfish with naturally elevated ambient levels of pathogenic Vp. (1) As a result, the development of diagnostic methods that can be used to monitor changes in pathogenic Vp. abundance in response to environmental stimulus and inform risk prediction models that can accurately identify when Vp. infection risk is elevated, is necessary to improve Vp. management strategies (25, 26, 37- 41).

A number of environmental Vp. surveillance efforts have been conducted in the U.S. and have provided a baseline understanding of seasonal and regional differences in Vp. abundance and ecology in coastal ecosystems (18, 25, 27). Most have employed Vp. detection methods that pool animals, usually in batches of 12, and use a serial dilution enrichment step for quantification (43). All currently FDA and NSSP approved detection methods use the thermolabile hemolysin (*tlh*) gene to spectate punitive Vp. from environmental samples (7, 43). However, the vast majority of environmental Vp. strains are not known human pathogens, making the use of total Vp. (*tlh*) as a surrogate for pathogenic Vp. strain abundance problematic (1, 18, 25, 27). The thermostable direct hemolysin (*tdh*) and the thermostable direct-related hemolysin (*trh*) genes, often present in pathogenic Vp. strains recovered from infected patients, are commonly used as genetic markers to discriminate the abundance of potentially pathogenic (*tdh*+ and/or *trh*+) Vp. in surveillance efforts (1, 18, 25, 27-29). While the quantification of the *tdh* and *trh* markers in the environment likely provides a better indicator of risk than total Vp. alone (28, 44- 48), the repeated isolation of environmental Vp. isolates that contain both the *trh* and *tdh* marker, but have not been associated with clinical infections, highlights their limitations as indicators of

pathogenic abundance and infection risk (28). Despite these limitations, the *tlh*, *tdh*, and *trh* markers, and the environmental conditions that influence their abundance, are commonly used in surveillance efforts to inform Vp. risk management strategies, and are the basis for the majority of current Vp. risk assessment models (1, 18, 28, 42, 44-49).

Vp. Ecology in the U.S.

Vp. can be found in seawaters, sediments and is commonly observed as constituent of the bacterial community of many marine species, such as the American Oyster (4, 9 -10, 16, 18). Studies have shown that total Vp. abundance in environmental oyster samples is positively correlated with temperature and, in general, abundance fluctuates seasonally, increasing in warmer months and decreasing in cooler months (2, 5, 25, 27, 50- 51). Vp. has a relatively wide thermal tolerance, but grows optimally at temperatures above 21°C, where it exhibits rapid exponential growth and has one of the fastest known doubling rates of any marine bacteria (1). Vp. growth slows to almost undetectable levels at temperatures below 10° C and cells can enter a viable but non-culturable state (53). Studies conducted in the U.S. Pacific Northwest detected Vp. in oysters at water temperatures above 15°C. In the Mid-Atlantic and Northeast U.S., Vp. was normally detected in shellfish samples at water temperatures above 9°C, although rarely at water temperatures as low as 4°C (25, 27, 54). Vp. is a halophile and can be found in waters with salinities between 5ppt and 35ppt. Acute rainfall reduces estuarine salinity, which has been associated with increased Vp. abundance (55- 56). However, a comparison of studies shows salinity can have a variable impact on Vp. abundance (2, 25, 27, 42, 51). While water temperature and salinity are considered the most important drivers of Vp. abundance (1), a number of other environmental parameters, including chlorophyll *a*, turbidity, suspended sediments, nutrients, and dissolved organic carbon, have all been associated to varying degrees

with Vp. abundance (18, 25, 27, 42, 57- 58). While this suggests a common array of environmental variables are key to understanding Vp. population dynamics and possibly risk (11, 19, 24, 40, 71, 72), the relationship between individual or multiple environmental parameters and Vp. abundance appears to be strongly variable between studies and study locations; limiting the transferability of data beyond the studied area (18, 25, 27).

The studies that have been conducted in regions where the *tdh* and/or *trh* markers are detected in a high enough frequency to statistically evaluate their relationship with environmental parameters have reported differences in the environmental conditions associated with total Vp. abundance and those associated with *tdh*+ and/or *trh*+ Vp. abundance (59). For example, Johnson et al. (58) and Zimmerman et al. (57) found that turbidity was correlated with *trh*+ and *tdh*+ Vp. abundance in oysters from the Gulf of Mexico, but not total Vp. abundance. Further, they observed a significant correlation to temperature and total Vp. abundance, but no correlation between temperature and *tdh*+ and/or *trh*+ Vp. abundance (57). Jones et al. observed the converse in Long Island Sound; with water temperature and salinity not significantly associated with total Vp. abundance, but significantly correlated with *tdh*+ and *trh*+ Vp. abundance (35). Flynn et al. reported a strong positive association between the *tlh*, *tdh*, and *trh* genetic markers and water temperature, however, when water temperatures exceed 22°C, no relationship was observed (37). Further, the relative abundance of the *tdh* marker to total Vp. was negatively associated with water temperature in colder waters and decreased exponentially as total Vp. increased (37). These observations suggest more complex ecological relationships can drive Vp. ecology and the abundance of pathogenic strains in the environment and present challenges for the use of total Vp. as a surrogate for pathogenic Vp. abundance (18, 37).

Vp. Ecology in the U.S. Northeast

The small number of studies that have been conducted in the Northeast have documented similar geographically mixed trends as those observed in other regions (5, 25, 27, 35). For example, Urquhart et al. found temperature, salinity, and chlorophyll *a* were useful predictors of elevated total Vp. abundance in the Great Bay Estuary in New Hampshire (25). Cox et al. found that in Rhode Island coastal waters and oysters Vp. levels tracked closely with water temperature (5). However, Jones et al. found no correlation between total Vp. and temperature or salinity in oysters collected from harvest areas in New York and Connecticut (35).

The detection rate of *tdh*+ and *trh*+ Vp. in surveillance efforts in the U.S. Northeast has been historically low (25, 27). For example, results from a 20-year dataset in Great Bay New Hampshire show that between 2007 to 2016 the *tdh* and *trh* pathogenicity markers were only detected in two samples in 2009 and were not detected again until 2015 (25). This low rate of detection in Northern New England is consistent with estimates in the U.S Food and Drug Administration's quantitative risk assessment for Vp. of an average 0.3% relative abundance of pathogenic Vp. to total Vp. in oysters from North Atlantic harvest areas and is a major reason why the risk of Vp. infection in the region was considered low (1). However, recent studies conducted in some southern New England harvest areas have documented a greater frequency of detection and higher relative abundance of *tdh*+ and *trh*+ Vp. than those predicated in the FDA quantitative risk assessment for Vp. in oysters (5, 35). For example, a 2012 study found that the relative percentage of *tdh*+ and *trh*+ Vp. to total Vp. in Rhode Island coastal waters was on average between 0.2% to 3.5% and 2.5% to 31.9%, respectively; with observations from individual harvest areas highly variable across a very limited spatial extent (5). Similarly, Jones et al. observed a greater than 50% detection rate of *tdh*+ and *trh*+ Vp. in oyster and clam samples

collected from Connecticut and New York harvest areas, as well as sample outcomes with relative levels of *tdh*+ Vp. to total Vp. well above the FDA estimated 0.3% (25). As the Cape Cod Peninsula serves as a major biogeographical barrier in the coastal waters of the Northwest Atlantic, with environmental conditions and species assemblages in harvest areas to the north of Cape Cod primarily influenced by the cool Labrador Current and environmental conditions and species assemblages in harvest areas to the South of Cape Cod largely influenced by the warm waters of the Gulf Stream (60), the observation of a higher relative abundance and rate of detection of *tdh*+ and/or *trh*+ Vp. in southern New England harvest areas as compared to northern New England harvest areas is generally consistent with standard temperature driven Vp. abundance and risk predication models due to the overall warmer water temperatures experienced in the southern portion of the region. However, increasing infections have been associated with oysters from harvest areas on both sides of Cape Cod (31), and individual harvest areas across the entire region can be highly influenced by localized hydrodynamics, with peak summer conditions in a number of northern New England harvest areas routinely reflecting an equal or higher estimated infection risk than their southern counterparts when standard risk assessment methods are applied (60). Recent insights into pathogenic strain emergence and analysis of clinical isolates from infections from the region suggest that the pathogenic strains of greatest human health significance may not be present in all harvest areas (28, 29), and differences in Vp. community composition at relatively limited spatial extents may contribute more to Vp. infection risk than the environmental conditions commonly considered conducive for Vp. bacteria (28), limiting the use of currently available risk assessment models to inform management efforts in the region (31). This suggests localized information is required in order to

accurately evaluate the factors that are leading to increased Vp. infection risk in the region inform appropriable scaled risk assessment and Vp. management strategies in the region.

RESEARCH OBJECTIVES

Our aim for this study was to produce information that can be used to assist with managing this emerging threat to shellfish consumer health and the Region's growing shellfish aquaculture industry. This thesis is separated into two parts.

Chapter II captures three years of Vp. surveillance data from three major harvest areas in the Massachusetts between 2015 and 2017. We evaluated the relationships between the absolute and relative abundance of total Vp. and the *tdh* and *trh* pathogenicity markers (potentially pathogenic Vp.) with water temperature, salinity, and chlorophyll *a* in the harvest areas.

Chapter II Objectives

The main objectives of this part of our research are:

1. Collect information on total and potentially pathogenic Vp abundance in MA harvest areas and evaluate Vp. levels vary between areas; and,
2. Identify environmental conditions correlated with increases in total and potentially pathogenic Vp abundance in MA harvest areas and evaluate how relationships differ between Massachusetts harvest areas with variable environmental conditions and historic illness occurrence.

We hypothesize that the relationship between total and potentially pathogenic Vp. abundance in oysters and environmental conditions will vary between harvest areas located on either side of Cape Cod. Furthermore, we hypothesize that we will observe differential relative

abundances of potentially pathogenic Vp. to total Vp. between and within study sites driven by differences in environmental conditions and Vp. population composition across harvest areas.

Chapter III presents an evaluation of 44 shellfish-borne Vp. cases linked to three distinct harvest regions in Massachusetts where information pertaining to the number and timing of Vp. cases, production levels, and environmental conditions in harvest areas on harvest dates implicated was available. We analyzed these data in an attempt to determine how observed variability in environmental conditions within and between the three harvest areas influenced Vp. infection risk in Massachusetts between 2014 and 2016. We also evaluated how our results compared with assumptions made in standard abundance based risk assessment methodology to determine the extent to which Vp. abundance modeling is a useful indicator of infection risk in Massachusetts. In addition, the sequence type of the clinical isolates in 39 of the 44 cases was evaluated to determine if there was variability in risk that may be associated with differences in Vp. communities between the studies areas.

Chapter III Objective

The main objectives of this part of our research are to:

1. Characterize trends in Vp. case occurrence and risk for individual Massachusetts harvest regions;
2. Identify how these trends vary between Massachusetts harvest areas and evaluate how trends in risk correspond with estimates in standard risk assessment methodology and environmental conditions; and,
3. Identify how differences in Vp. community composition may influence risk at limited spatial extents.

We hypothesize that we will observe differences in risk and the environmental conditions associated with infection occurrence between harvest areas. Differences will be primarily due to variability in Vp. community composition as indicated by Vp. surveillance and clinical isolate analysis. In particular, we hypothesize the primary driver of risk will be the extent ST36 is established in a particular areas.

Our research explores previously uninvestigated spatial and temporal interactions related to total and potentially pathogenic Vp. abundance, environmental conditions, Vp community composition, and illness occurrence; therefore, our work offers an original effort to evaluate how differences in these factors can influence illness occurrence and Vp risk at limited spatial and temporal extents, and how the use of surveillance data and epidemiological reporting can be applied to Vp. risk prevention and risk assessment methodology.

CHAPTER II

RELATIONSHIPS BETWEEN ENVIRONMENTAL CONDITIONS AND SEASONAL LEVELS OF TOTAL AND PATHOGENIC *Vibrio parahaemolyticus* IN OYSTERS (*Crassostrea virginica*) IN MASSACHUSETTS

INTRODUCTION

Vibrio parahaemolyticus (Vp.*)* is a naturally occurring human pathogenic bacterium and the leading cause of bacterial derived seafood poisoning in the U.S. (12-13). Human gastric infections from pathogenic Vp. exposure can cause self-limiting gastroenteritis, and in rare cases septicemia (14, 17). Vp. is a halophile and commonly found in the same coastal waters where shellfish, such as the eastern oyster (*Crassostrea virginica*), are cultured (2, 55-56). Vp. cells can attach to particulates and other organisms in the marine environment and be concentrated in molluscan shellfish digestive tissues through particulate uptake via normal filter feeding activities (1, 2). Due to this filter feeding behavior, the overlap in habitat, and consumer trends favoring raw consumption, oysters are the most commonly reported vector in Vp. gastric infections in the U.S. (1, 3- 6).

Environmental Vp. abundance is strongly correlated with temperature and generally demonstrates a seasonal trend with the highest levels observed in summer months (1). Vp. has one of the fastest observed doubling times of all bacteria and is capable of rapid exponential growth at temperatures above 21°C (1). Growth slows to almost undetectable levels at temperatures below 10°C, where most cells enter a viable but non-culturable state (53). Due to the association with Vp. abundance and temperature, the highest levels of Vp. bacteria are estimated to be in the warm coastal waters of lower latitudes, where Vp. is detectable year round

and Vp. derived gastroenteritis in humans have been historically common (25, 27, 61-62). In the cool coastal waters characteristic of temperate regions, such as New England, Vp. is seasonally detectable during the warm summer months, becoming non-detectable during cold winter months (5, 25, 27, 35, 64-69). Gastric Vp. infections associated with the consumption of oysters harvested from New England coastal waters have historically been sporadic in nature, presumably due to a generally low abundance of pathogenic Vp. strains in the cool waters of the Northwest Atlantic (1). Over the last decade, however, increasing numbers of Vp. outbreaks associated with the consumption of oysters harvested from the region have been reported (5, 25, 27, 28, 30, 35), and in particular in Massachusetts, where recurrent seasonal outbreaks resulting in harvest areas closures, product recalls, and the implementation of costly control measures on the harvesting and handling of oysters during summer months has led to significant impacts on the state's growing aquaculture industry (25, 27, 30, 31).

A combination of factors, such as introduced and ecosystem establishment of non-endemic pathogenic strains, increased summertime production and consumption of raw shellfish, and climate related changes causing warmer sea surface temperatures and more variable salinities resulting in increasingly favorable conditions for Vp have all been attributed to the recent increase in infections in the region (25, 27, 30, 31, 35). The growing public health and economic burden associated with managing shellfish consumer Vp. risk in New England have made the development of diagnostic methods and predictive models that can accurately characterize pathogenic Vp. infection risk and inform growers and managers of periods of increased risk a major priority for the region (7, 25, 27).

A considerable amount of environmental surveillance aimed at understanding the biotic and abiotic factors that influence total Vp. abundance has been conducted in the U.S. (18, 25, 27,

42). Beyond temperature, a number of environmental parameters, including salinity, chlorophyll *a*, turbidity, suspended sediments, nutrients, and dissolved organic carbon, have been associated with Vp. abundance (2, 5, 18, 25, 27, 42, 50- 51). While this suggests a common array of measured variables may be key to understanding Vp. population dynamics, the relationship between individual or multiple environmental parameters with Vp. abundance is strongly variable between regions and in some cases on a harvest area by harvest area basis, limiting the transferability of study data beyond the studied area (25, 27). Based on the observed differences in the environmental conditions that correlate with Vp. abundance on relatively limited spatial extents, it is likely the development of models based on information by surveillance efforts conducted at a limited number of sites and locations, could result in erroneous assumptions when applied at a regional or even statewide basis (25, 27). This suggests a more nuanced approach is required to accurately evaluate the environmental conditions that favor pathogenic variants and increase Vp. infection risk to inform risk assessment strategies. This would be particularly true in regions or states with significant variability in environmental conditions between harvest areas such as Massachusetts (60).

The use of Vp. surveillance data and total Vp. abundance models to inform mitigation strategies assumes a connection between total Vp. abundance and infection risk (1, 25); however, there is debate on how total Vp. abundance in shellfish at harvest actually relates to infection occurrence or risk (25, 28, 36, 70). There are a number of regulatory reference thresholds for total Vp. in shellfish that associate total Vp. abundance with consumer safety, including a U.S. requirement that levels of Vp. in post-harvest processed shellfish be below 30 MPN g^{-1}; (7), and the Canada Seafood Inspection Service limit for Vp. in shellfish of 100 MPN g^{-1}; (71). However, currently there is no regulatory threshold for background Vp. levels in shellfish meats in the U.S

(7). This is likely because the use of total Vp. as an indicator for infection risk is considered problematic, as the majority of environmental Vp. strains are not known human pathogens and current accepted detection methods lack the nuance to accurately differentiate the abundance of pathogenic to non-pathogenic Vp. strains in the environment (1, 18, 25, 27).

Two hemolysin genes (*tdh* and *trh*), present in the majority of Vp. isolates recovered from infected patients, are often used as markers to discriminate the abundance of potentially pathogenic strains in surveillance efforts and likely provide a better indicator of risk than total Vp. (1, 18, 25, 27-29). However, due to the rapid emergence of Vp. as a public health concern in New England, limited information related to the distribution and abundance of *tdh*+, and *trh*+ Vp., in shellfish harvest areas in the region has been collected that can help inform Vp. risk assessment models and more nuanced management strategies (25, 27, 35).

A number of recent Vp. abundance modeling efforts have been conducted in New Hampshire Coastal waters, however the rate of detection of *tdh*+ and *trh*+ Vp. in surveillance efforts in Northern New England is historically rare (25, 27). A number of recent studies conducted in southern New England harvest areas have documented a greater frequency of detection and higher relative abundance of *tdh*+ and *trh*+ Vp. than those observed in the northern half of the region. For example, a 2012 study found that the relative percentage of *tdh*+ and *trh*+ Vp. to total Vp. in Rhode Island coastal waters was on average between 0.2% to 3.5% and 2.5% to 31.9%, respectively; with observations from individual harvest areas highly variable across a very limited spatial extent (5). Similarly, Jones et al 2014 observed a greater than 50% detection rate of *tdh*+ and *trh*+ Vp. in oyster and clam samples collected from Connecticut and New York harvest areas, as well as sample outcomes with relative levels of *tdh*+ Vp. to total Vp. well above the estimated 0.03% (35).

As the Cape Cod Peninsula serves as a major biogeographical barrier in the coastal waters of the Northwest Atlantic, with environmental conditions and species assemblages in harvest areas to the north of Cape Cod primarily influenced by the cool Labrador Current and environmental conditions and species assemblages in harvest areas to the South of Cape Cod largely influenced by the warm waters of the Gulf Stream (60), the observation of a higher relative abundance and rate of detection of *tdh*+ and/or *trh*+ Vp. in southern New England harvest areas as compared to northern New England harvest areas is generally consistent with standard temperature driven Vp. abundance and risk predication models due to the overall warmer water temperatures experienced in the southern portion of the region. However, individual harvest areas on either side of the Cape Cod peninsula can be highly influenced by localized hydrodynamics, and in a number of northern New England harvest areas the routine and/or episodic environmental conditions often reflect an equal or higher estimated infection risk than their southern counterparts when standard risk assessment methods are applied (60). Massachusetts' straddles this biogeographical barrier, likely limiting the utility of a generalized Vp. risk assessment model for the state, and driving the need for the collection of robust baseline information to inform effective Vp. management strategies. To support Vp. management efforts in Massachusetts we conducted routine Vp. surveillance in three major harvest areas in the state between 2015 and 2017 and evaluated the

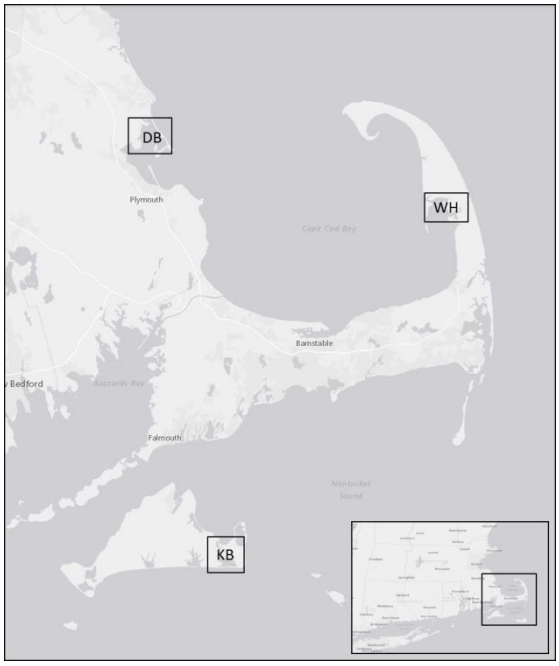


Fig 1. Massachusetts, USA, with harvest are Wellfleet Harbor (WH), Duxbury Bay (WCCB) and Katama Bay (KB).

relationships between the observed absolute and relative abundance of total Vp. and the *tdh* and *trh* pathogenicity markers (potentially pathogenic Vp.) with water temperature, salinity, and chlorophyll in the harvest areas. The main research objectives of this study are to advance the understanding of the relationships between total and potentially pathogenic Vp. and environmental parameters in Massachusetts, and second, to determine how these relationships differ between harvest areas across Massachusetts. We hypothesize that we will observe variability in the relationship between total Vp. and potentially pathogenic Vp. abundance in oysters and environmental conditions between harvest areas located on either side of Cape Cod. Furthermore, we hypothesize that we will observe differential relative abundances of potentially pathogenic Vp. to total Vp. between and within study sites driven by differences in environmental conditions and Vp. population compositions across harvest areas.

METHODS

Study Sites

The three areas evaluated in the study included Katama Bay in the Town of Edgartown Massachusetts on Martha's Vineyard, Duxbury Bay (DB) and Wellfleet Harbor (WH) (Fig 1.). While, all three sites are similarly affected by regional climatological changes that result in seasonal shifts in temperature, salinity, and primary productivity, differences in tidal and current dynamics result in variability in environmental conditions and ecosystem dynamics between the sites (60). Katama Bay is a tidal inlet system with subtidal oyster culture and limited daily tidal variability (~1m). Duxbury Bay and Wellfleet Harbor are both open bay systems with significant daily tidal variability (3-3.5m).

Environmental Sampling

Samples, each consisting of 12-15 hatchery-reared oysters (*Crassostrea virginica*), were collected by commercial harvesters or Massachusetts Division of Marine Fisheries personnel on a semi routine basis between May and October 2015-2017. Within 0-1hr of exposure to ambient air, samples were placed in an insulated cooler with wet ice. Samples were transported directly to laboratory facilities in MA or transferred to ice packs and overnight shipped to laboratory facilities in New Hampshire. Internal oyster temperature was measured upon arrival at laboratory facilities by partially shucking the oysters and inserting a stem thermometer. Any samples with internal temperatures >10°C were not included in analysis. Oysters in each sample were cleaned, shucked, homogenized and processed for analysis according to FDA BAM (54) protocols. The homogenate was diluted using a 3-tube, five dilution or more series and incubated at 35-37°C. For RT-PCR enumeration, a 1 mL sample from turbid tubes was boiled to lyse cells, the debris cleared by centrifuging, and 2 ul of the lysate analyzed. The tiered analysis first employs the FDA/NSSP MPN RT-PCR procedure (72) to quantify total Vp. using the Vp. species-specific marker *tlh* and an internal amplification control (IAC). Positive *tlh* samples were then examined in duplex for the presence of hemolysin containing strains using primers and probes specific to *tdh/trh* (72). To adjust for variability in the detection threshold across labs, sample results for *tlh*, *trh* and *tdh* that were <3 MPN/g were treated as non-detects in the analysis. Analysis of environmental isolates was conducted by quadrant streaking turbid enrichments on Vibrio CHROMAgar (CHROMagar, Paris, France) and sequencing the genome of individual Vp isolates via Illumina technology. Sequences of seven housekeeping loci were isolated from the resulting data for each isolate and used to compare with allele combinations in previously

reported strains in the MLST database. The level of detection (LOD) for all three genetic markers (*tlh, tdh, trh)* was 0.48 log MPN/g.

Environmental parameters, including water temperature (°C), salinity (ppt), and chlorophyll *a* (ug/l) were measured for the Duxbury Bay and Wellfleet Harbor sites using YSI EXO2 multi-parameter data sondes (YSI, Inc. Yellow Springs, OH), owned and maintained by The Barnstable County Cooperative Program. In Katama Bay, chlorophyll (ug/L) was measured using a Cyclops data logger with Turner fluorimeter probe. Water temperature (°C) and conductivity were measured via an Onset Computer HOBO UA-002-08 Temperature Pendant and U24-002-C Salinity Data Logger (Onset Computer Inc. Onset, MA), respectively. Upon retrieval, conductivity was converted to salinity with the HOBOware Conductivity Assistant (Version 2.1) that employs a non-linear temperature coefficient generated using the PSS-1978. Discrete samples from Katama Bay analyzed via a bench top ThermoFisher Scientific filter fluorimeter for chlorophyll *a* (ug/l) and YSI ProDSS for temperature, and conductivity were used to verify in-situ data. All in-situ parameters were measured at 15-minute intervals.

Statistical Analysis

Median total and potentially pathogenic Vp. levels are reported based on log-transformed values from all sample outcomes above the level of detection. Differences between distributions of abundances were evaluated by Mann-Whitney rank sum tests. Spearman correlation was used to assess the association between total and potentially pathogenic Vp. levels and environmental parameters. The statistical significances of observed differences and associations were determined using an alpha level of 0.05. These nonparametric tests were selected as generally applicable given a high proportion of observations below the LOD for the *tdh* and *trh* gene targets. When conducting group level comparison and correlation analysis half the limit of

detection (LOD) was substituted for sample outcomes below the LOD. All analyses were conducted using StatPlus 3 7.1.0 (AnalystSoft Inc., Alexandria, VA).

RESULTS

Statewide Detection and Vp. Levels.

A total of 273-oyster samples were collected over the three-year study period. Total Vp. (*tlh*) was detected in 205 of 273 (75%) samples with a median level and range of 1.08 (range, 0.48 to 4.18) log MPN/g. This is a lower median level than reported for Connecticut oysters (2.18 log MPN/g) (35) and slightly higher than those reported for New Hampshire (0.86 log MPN/g) (25), but comparable to levels (10^3–10^4 MPN g^{-1}) found in shellfish from other coastal regions (58, 2, 3). The *tdh* marker was detected in 48 of 273 (18%) samples with a median level and range of 0.52 (range, 0.48 to 2.97) log MPN/g. The *trh* marker was detected in 95 of 273 oyster (34%) samples with a median level and range of 0.56 (range, 0.48 to 2.96) Log MPN/g. The median *tdh* and *trh*+ Vp. levels we observed were substantially higher than the median *tdh* and *trh* levels reported for Connecticut oysters (-0.44 Log MPN/g) (35). Although, it is important to note that the study conducted on Connecticut oysters had a lower limit of detection (-0.52 Log MPN/g) than that used in our study (0.52 Log MPN/g). It is possible that the use of a lower LOD would have resulted in more comparable median levels of *tdh*+ and *trh*+ Vp between the two studies. The maximum level of total Vp. observed in oysters in this study (4.18 Log MPN/g) was slightly higher than that reported for Connecticut and Rhode Island oysters (~3.95 Log MPN/g). However, the maximum level of *tdh*+ (2.97 Log MPN/g) and *trh*+ Vp. (2.97 Log MPN/g) observed in oysters in this study were over an order of magnitude higher than the maximum *tdh*+ and *trh*+ Vp. levels reported for Connecticut oysters (1.63 and 1.88 Log MPN/g, respectively),

and only comparable to a single value reported for one coastal pond (CP2) in Rhode Island (5, 35).

	Gene Detection Frequency			*tlh*, *tdh*, *trh* Median Level (Range) Log MPN/g of Samples Above LOD		
	tlh	*tdh*	*trh*	*tlh*	*tdh*	*trh*
Duxbury Bay	98/123	27/123	57/123	1.17 (0.48 to 4.18)	0.57 (0.48 to 1.63)	0.87 (0.48 to 2.38)
Katama Bay	84/123	18/123	26/123	0.96 (0.48 to 3.96)	0.48 (0.48 to 2.97)	0.48 (0.48 to 2.97)
Wellfleet Harbor	23/27	3/27	12/27	0.96 (0.48 to 2.45)	0.48 (0.48 to 0.57)	0.71 (0.48 to 1.97)
Overall	205/273	48/273	95/273	1.08 (0.48 to 4.18)	0.52 (0.48 to 2.97)	0.56 (0.48 to 2.97)

Table 1 Detection frequency, median, and range of *tlh*, *trh*, and *tdh* abundance from oysters at samples sites 2015-2017

Site Specific Detection and Levels

The number of samples collected from Duxbury Bay and Katama Bay (DB, n=123; Katama Bay, n= 123) varied from Wellfleet Harbor (n=27). The significantly lower number of samples collected from Wellfleet Harbor was the result of logistical challenges associated with access to the site being tidally dependent. We observed differences in the rate of detection and median and maximum total Vp. levels between sites. Total Vp. was detected in 98/123 (79%) of samples from DB, 84/123 (68%) from Katama Bay, and 23/27 (96%) from Wellfleet Harbor. The highest median and maximum total Vp. level was observed in Duxbury Bay and total Vp. levels (P= 0.02) were significantly higher in Duxbury Bay than in Katama Bay. No other effects from sampling location on total Vp. levels were observed. The maximum total Vp. levels in Duxbury Bay (4.18 Log MPN/g) and Katama Bay (3.96 Log MPN/g) were similar, with both approximately an order of magnitude higher than the maximum level observed in Wellfleet Harbor (2.45 Log MPN/g). *tdh*+ Vp. was detected in 27/123 (22%) of samples from Duxbury

Bay, 18/123 (15%) from Katama Bay, and 3/27 (11%) from Wellfleet Harbor. There was no effect of sampling location on *tdh*+ Vp. levels, and median levels between the three sites were similar (Table 1). However, the maximum *tdh*+ Vp. level for Katama Bay (2.97 Log MPN/g) was more than an order of magnitude higher than that observed for Duxbury Bay (1.63 Log MPN/g) and two orders of magnitude higher than that observed in ECC (0.57 Log MPN/g). The genetic marker *trh* was detected in 57/123 (46%) of samples from Duxbury Bay, 26/123 (21%) from Katama Bay, and 12/27 (44%) from Wellfleet Harbor. Levels of *trh*+ Vp. were significantly higher in Duxbury Bay than in Katama Bay (P=0.03) and Wellfleet Harbor (P= >0.001) and between Wellfleet Harbor and Katama Bay (P=0.03). While median *trh*+ Vp. levels were lowest in Katama Bay, the maximum *trh*+ Vp. level observed for Katama Bay (2.97 Log MPN/g) and Duxbury Bay (2.38 Log MPN/g) were approximately an order of magnitude higher than that reported for Wellfleet Harbor (1.97 Log MPN/g). The higher maximum levels of *tdh*+ and *trh*+ Vp. in Katama Bay followed by Duxbury Bay are consistent with reported trends for Vp. infection risk between Massachusetts harvest areas (Schillaci et al 2020a). Gene occurrence and descriptive statistics for all samples and at individual sites are presented in Table 1.

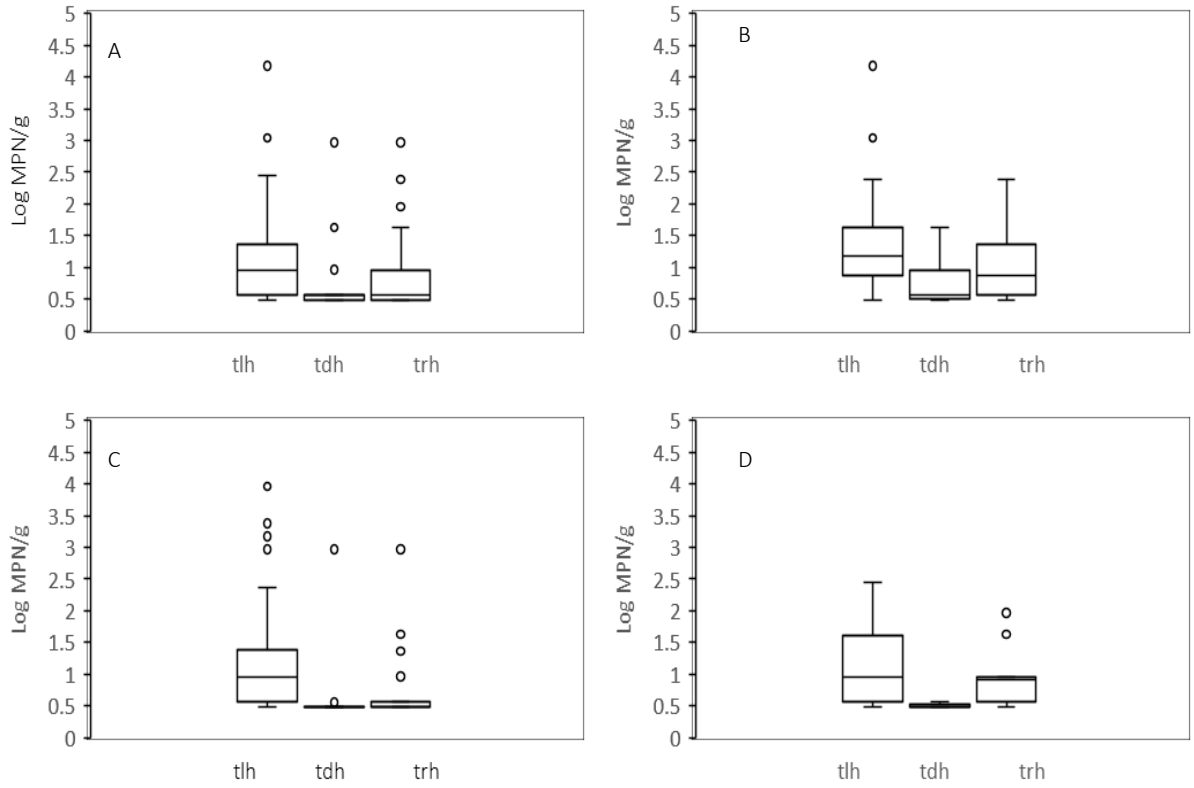


Fig 2. Levels of total Vp. *tdh*, and *trh* log10 MPN/g in oysters at all harvest sites. A= Overall, B= Duxbury Bay, C=Katama Bay, D= Wellfleet Harbor. Hollow circles represent outliers; vertical lines illustrate ICQ range; bold horizontal bars represent

Relative abundance of potentially pathogenic Vp. to total Vp.

Statewide, the mean relative abundance and range of *tdh*:*tlh* and *trh*:*tlh* was 7.1% (0-100%) and 17.1% (range, 0-100%), respectively. Similar to observations in differences between maximum *tdh*+ Vp. levels between sites, the highest average relative abundance of *tdh*:*tlh* was observed at the Duxbury Bay (8.2%) and Katama Bay (7.3%) sites. This is consistent with the observation of a higher maximum level of *tdh*+ Vp. in both harvest areas. The average relative abundance and range for Wellfleet Harbor was similar to that observed in other New England harvest areas (Cox) and those reported in other regions (Flynn….). The average relative abundance and range of *trh*:*tlh* for Katama Bay and Wellfleet Harbor were similar and both

slightly higher than those reported for Rhode Island. The average relative abundance and range of *trh*:*tlh* for Duxbury Bay was the highest of the three study areas. These values, in particular those observed for average *tdh*:*tlh* abundance, are substantially higher than the average relative abundance of *tdh*:*tlh* (1.1%) and *trh*:*tlh* (12.1%) observed in Rhode Island (Cox) and far higher than that predicated for the North Atlantic region in the 2005 FDA quantitative Vp. risk assessment for oysters (FDA, 2005).

		mean % (range)
Area	*tdh:tlh*	*trh:tlh*
Duxbury	8.2% (0-100)	23.8% (0-100)
Katama	7.3% (0-100)	10.6% (0-100)
Wellfleet	0.8% (0-15)	14.3% (0-100)
Overall	7.1% (0-100)	17.1% (0-100)

Table 2. Percentages of *tdh*+ and *trh*+ Vp. relative to total Vp. (*tlh*) for all sites

Association of total and potentially pathogenic* Vp. *with environmental parameters.

Environmental parameters at all sites showed similar trends across years (Figures 3-5). Across all sampling events, observed water temperatures ranged from 14.2 to 28.0°C (median, 21.7°C), salinity ranged from 25.2 to 32 ppt (median, 29.9 ppt), and chlorophyll *a* ranged from 2.2 to 24.3 ug/L (median, 7.3 ug/L) (Table 3). Water temperatures and salinities observed in Duxbury Bay were significantly lower than those observed in Wellfleet Harbor (P= <0.001 and P= <0.035, respectively) and Katama Bay (P= <0.001 and (P= <0.001, respectively). Chlorophyll *a* levels observed in Wellfleet Harbor were significantly higher than those observed in Katama Bay (P= <0.001) and Duxbury Bay (P=<0.001). No other significant or marginally non-significant differences in water temperatures or other environmental parameters were observed between sites.

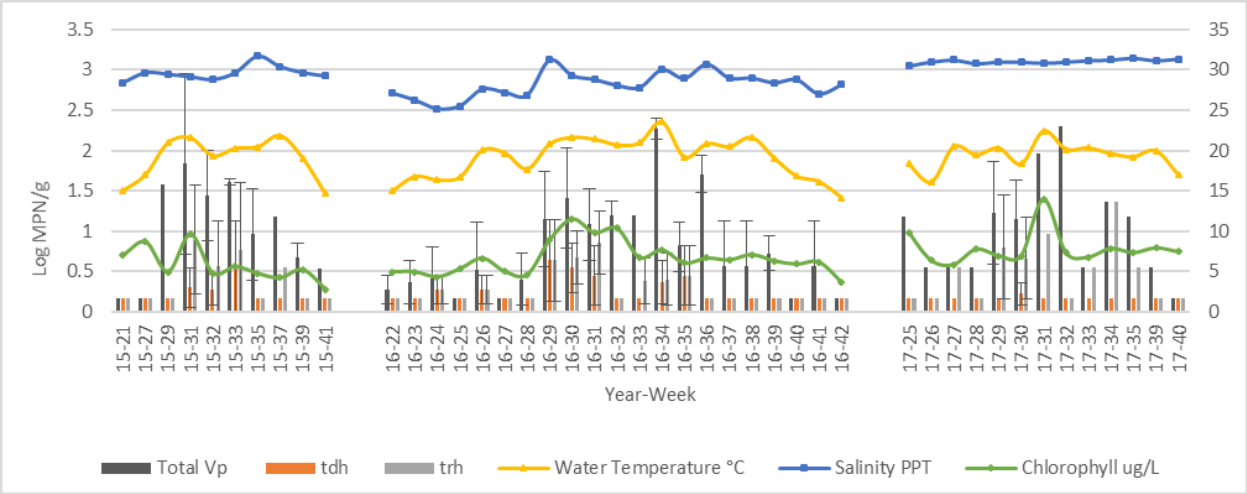


Fig 3. Temporal variation (year-week) of environmental parameters (right axis) and mean Total Vp., *tdh*, and *trh* levels (left axis) in oyster samples collected from Duxbury Bay 2015-2017. Error bars represent the standard deviation for weeks with more than one sampling occasion. For sample outcomes <LOD, 1/5 the LOD was substituted.

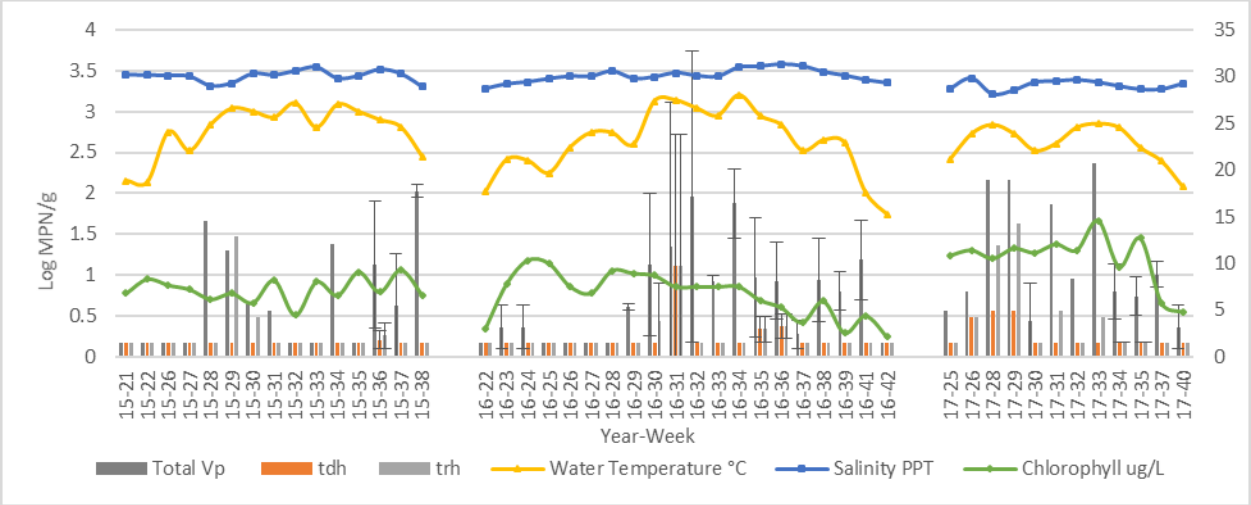


Fig 4. Temporal variation (year-week) of environmental parameters (right axis) and mean Total Vp., *tdh*, and *trh* levels (left axis) in oyster samples collected from Katama Bay 2015-2017. Error bars represent the standard deviation for weeks with more than one sampling occasion. For sample outcomes <LOD, 1/5 the LOD was substituted.

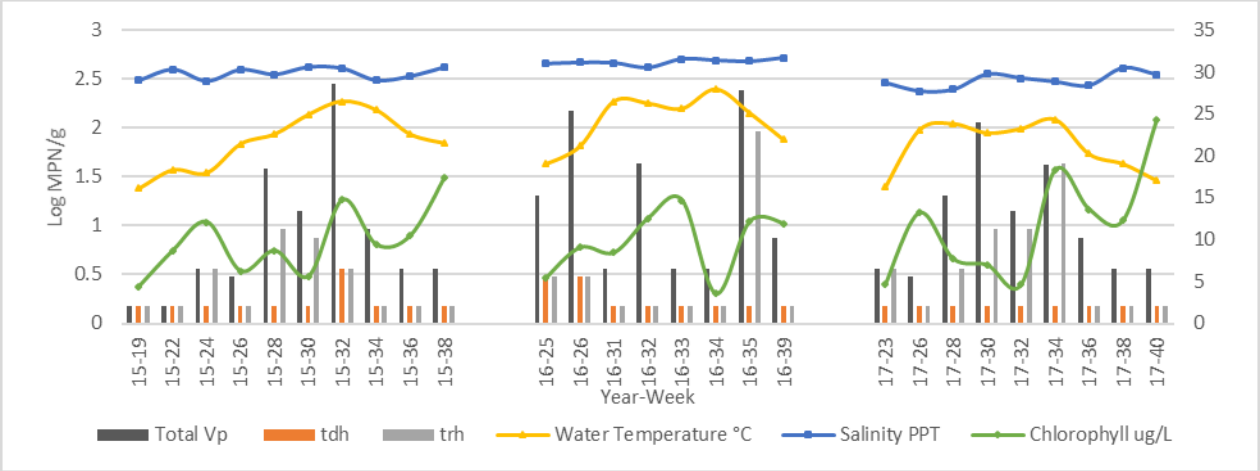


Fig 5. Temporal variation (year-week) of environmental parameters (right axis) and mean Total Vp., *tdh*, and *trh* levels (left axis) in oyster samples collected from Wellfleet Harbor 2015-2017. For sample outcomes <LOD, 1/5 the LOD was substituted.

	Duxbury Bay	Katama Bay	Wellfleet Harbor	Overall
Water Temperature (^{O}C)	20.3 (14.2-23.6)	24.6 (15.3-28.1)	22.6 (16.2-28)	21.7 (14.2-28.0)
Salinity (PPT)	29.3 (25.2-32)	30.2 (28.2-31.5)	30.3 (27.7-31.7)	29.9 (25.2-32)
Chlorophyll *a* (ug/L)	6.8 (2.9- 14)	7.3 (2.2-15.6)	9.4 (3.6-24.3)	7.3 (2.2-24.3)

Table 3. The median and range of environmental parameters observed during sampling events at the three study sites individually and combined.

The extent levels of total Vp., *tdh*+ Vp., and *trh*+ Vp. correlated with individual environmental parameters varied widely between sites (Table 4). Consistent with observations from New Hampshire and Rhode Island, water temperature showed the greatest correlation with total Vp. levels for combined data (Spearman's correlation coefficient (r_s) = 0.23, P =< 0.001) and at individual sites (Duxbury Bay, (r_s) = 0.55, P =< 0.001; Katama Bay, (r_s) = 0.31, P =< 0.001; Wellfleet Harbor, (r_s) = 0.43, P =0.024). Duxbury Bay was the only site where total Vp. and salinity (r_s) = 0.32, P =< 0.001) were significantly correlated. This is likely the result of the

greater range of salinities observed during the study period in Duxbury Bay than the other sites. Significant correlations between chlorophyll *a* levels and total Vp. observed for combined data (r_s) =0.21 P =<0.001) and Duxbury Bay (r_s) = 0.39, P =< 0.001), but not for other sites. Correlations between *tdh*+ Vp. and environmental parameters were limited. Levels of *tdh*+ Vp. in Duxbury Bay significantly correlated with water temperature (r_s) = 0.32, P =< 0.001) and chlorophyll *a* (r_s) = 0.35, P =< 0.001). Levels of *tdh*+ Vp. in Katama Bay significantly correlated with salinity (r_s) = 0.36, P =< 0.001). Levels of *trh*+ Vp. significantly correlated water temperatures and salinity in Duxbury Bay (r_s) = 0.26, P =< 0.001 and (r_s) = 0.24, P = 0.007), respectively) and Katama Bay (r_s) = 0.26, P =0.004 and (r_s) = 0.27, P =0.003), respectively). Significant correlations between chlorophyll *a* and *trh*+ Vp. levels observed for combined data (r_s) =0.29 P =<0.001) and Duxbury Bay (r_s) = 0.38, P =< 0.001). No other significant or marginally nonsignificant correlations were observed. Spearman's correlation coefficient for all environmental parameters and levels of total, *tdh*+, and *trh*+ Vp. for individual sites and data from all sites combined are presented in Table 4.

	Log_{10}-*tlh* (MPN/g)				Log_{10}-*tdh* (MPN/g)				Log_{10}-*trh* (MPN/g)			
	Overall	DB	KB	WH	Overall	DB	KB	WH	Overall	DB	KB	WH
Temp °C	0.23	0.55	0.31	0.43	0.1	0.32	0.17	0.06	0.06	0.46	0.26	0.13
Sal. PPT	0.11	0.32	0.02	0.18	0.08	0.03	0.36	0.27	0.10	0.24	0.27	-0.14
Chl a ug/L	0.21	0.39	0.005	0.12	0.09	0.35	-0.21	0.02	0.15	0.38	-0.12	-0.17

Table 4. Spearman's correlation coefficients for log transformed *tlh*, *tdh*, and *trh* values (MPN/g) and environmental parameters. Greyed boxes represent significant correlations with a CI= .05%.

Temporal Trends in Total and Potentially Pathogenic* Vp *levels in Oysters.

Overall trends in the seasonal occurrence and maximum levels of total and potentially pathogenic Vp. observed during the study, were comparable to those reported in oysters from other harvest areas in the region (Figures 3-5). (5, 25, 35). Trends were variable between sites, but in general total and potentially pathogenic Vp. levels were highest between July 1 and Sept 15, which is consistent with the observed peak Vp. risk period in the state and observations from other studies in the region (Chapter 3, 25, 73, 74).

DISCUSSION

Consistent with the results of previous studies conducted in the Northeast and other regions, our results demonstrate significant associations between water temperature and total Vp. levels in Massachusetts oysters at all three sites (5, 25, 27, 37,73, 74). The observed relationship between salinity and Vp. abundance in Massachusetts also reflects observations of previous studies (18), showing variable associations in areas with a fairly narrow range of salinity. While total Vp. abundance generally followed seasonal increases in water temperature, the association between Vp. abundance and mid-season variability in water temperature was limited at most sites with the exception of Duxbury, which was the only site where significant correlations between water temperature and all three genetic markers were observed. This observation is consistent with that of a recent study conducted in Washington State that found the generally positive correlation between total and *tdh*+ and *trh*+ Vp. abundance and water temperature that became non-linear when water temperatures exceeded 22°C (37). Thus, because temperatures in

Duxbury Harbor were the coolest of the three study areas and rarely exceeded 22°C, even in the peak of summer, the relationship remained significant.

Despite the positive correlation with water temperature and total Vp., at all sites we observed non-detectable total and *tdh*+ and *trh*+ levels in oyster samples collected during the peak of summer, as well as significant short term variability in Vp. abundance at individual sites with no concurrent change in measured environmental parameters. For example, we observed background total Vp. levels in Katama Bay fluctuate from non-detectable levels to 3.96 Log MPN/g within 24 hours, with no difference in water temperature beyond normal daily fluctuation. As total Vp. levels of 3.96 Log MPN/g are considered to represent levels of concern for human health (1), the significant variability within a 24 hour period calls into the question the utility of routine Vp. surveillance to capture trends in Vp. abundance that may indicate periods of increased risk. We also observed significant variability in Vp. levels between replicate samples collected from the same cage at the same time, suggesting there can be a high degree of variability in Vp. levels on an oyster to oyster level that is completely unrelated to differences environmental conditions. A number of other studies have reported similar levels of background variability (57, 75- 76) as well as oyster-to-oyster variability in Vp. abundance within samples (75-76). If major daily fluctuations in Vp. abundance and oyster-to-oyster variability is common, accurately capturing short term trends in Vp. abundance within harvest areas would require a sampling frequency and sample size to that is not practical for most State programs. Therefore, Vp. surveillance likely is best suited to evaluating differences in baseline risk between harvest areas, as opposed to use as a real time trigger for Vp. controls or harvest area closures.

Despite the observed positive relationship between water temperature and Vp. levels, a number of our findings were counter to standard temperature based risk assessment methodology

(5, 25, 27, 78). For example, generally you would estimate the lowest Vp. abundance and risk of the three study areas to be in Duxbury Bay due to the significantly cooler water temperatures (1). Yet, Duxbury Bay had the highest median total and *tdh*+ and *trh*+ Vp. levels of the three study sites, as well as the highest average relative abundance of *tdh*+ and *trh*+ Vp. to total Vp. Studies conducted in the PNW have also reported that the areas and periods where the highest median total, *tdh*+ and *trh*+ Vp. abundance are observed are often not associated with the periods or areas where the highest water temperatures are observed (79). Recent insights into pathogenic strain emergence in the Northeast suggest that differences in bacterial populations between harvest areas may play a larger role in enhanced disease risk than environmental conditions traditionally considered conducive for rapid Vp. growth (28). This hypothesis does fit a number of observed trends associated with epidemiological reporting in Massachusetts (Chapter 3). For example, while both Duxbury Bay and Wellfleet Harbor are located to the north of Cape Cod Bay, we observed more similarity in the absolute and relative abundance of total and potentially pathogenic Vp., and the results of correlation analysis, in Duxbury Bay and Katama Bay than for Wellfleet Harbor and Duxbury Bay. This may be in part due to similarities in the pathogenic Vp. communities in Katama Bay and Duxbury Harbor, with the majority of Vp. infections in both areas associated with strains from the non-endemic ST36 complex (28, Chapter 3). Trends in infection risk we observed when evaluating epidemiological reporting data from Massachusetts (Chapter 3) also appear to follow closely with trends in maximum *tdh*+ Vp. values we observed in our three study areas. For example, the maximum recorded value for *tdh*+ Vp. in Katama Bay was 2.97 log10MPN/g, which is roughly an order of magnitude higher than the maximum recorded value for *tdh*+ Vp. in Duxbury Bay of 1.63 log10MPN/g, and two orders of magnitude higher than the maximum recorded value for *tdh*+ Vp. in Wellfleet Harbor of 0.57 log10MPN/g.

These results are consistent with trends in differences in the observed risk between Massachusetts harvest areas between 2014 and 2016 where the average risk per serving between 2014 and 2016 in the regions containing our study areas was 2.9 cases/100,000 oysters in Katama Bay, 0.7 cases/100,000 oysters in Western Cape Cod Bay where Duxbury Bay is located, and 0.37 cases/100,000 oysters in Wellfleet Harbor. We also observed higher rates of detection of *tdh*+ Vp. and higher relative percentages of *tdh*+ Vp. to total Vp., in Katama Bay and Duxbury Bay, which are both located in areas where higher infection risks were observed (Chapter 3).

CONCLUSIONS

In summary, the current study examines the relationship between Vp in oysters and various independent environmental parameters in Massachusetts harvest areas. Overall our results suggest limitations to estimating Vp. risk in Massachusetts on a statewide basis from surveillance conducted at a limited number of sites and at a limited sampling frequency as well as challenges for the development of localized risk assessment models based on total, *tdh*+ and *trh*+ Vp. However, our results did provide useful information on differences in the baseline absolute and relative abundance of total and potentially pathogenic Vp. levels, and information on trends in their relationship to environmental parameters, that can be used to inform Vp. management strategies in Massachusetts. For example, while there were slight differences between sites, the annual occurrence of when total, *tdh*+ and *trh*+ Vp. were first detected and peaked were similar enough between harvest areas to inform the establishment of a statewide seasonal control period between late May and early October. While focusing on predictable trends and variables to establish seasonal controls may mean that when areas experience short-

term weather fluctuations the controls in place may appear to be overly cautious, considering the observed short-term variability in Vp. abundance within harvest areas, adjusting controls to match real time conditions would generally be incompatible with the legal processes required to implement and enforce regulatory controls, and the practical aspects of notifying harvesters and enforcement officers that new controls are in place. With enough data it may be possible to develop nuanced controls and risk prediction models that address the type of short-term variability we observed, but maintain a level of predictability. For example, if surveillance data were collected in a manner that could indicate if there is a consistent daily period of low Vp. abundance (i.e. between sunrise and 10 AM) the State or harvesters could establish a seasonal daily harvest window, or tiered controls based on daily trends. While our data scratched the surface at correlating Vp. abundance with observed patterns in infection risk, until there is a greater understanding of the correlation between total, *tdh*+ and *trh*+ Vp. abundance in shellfish and illness occurrence, it is likely that Vp. surveillance and risk modeling will continue to provide the greatest value for use to describe baseline trends to inform seasonal Vp controls. Advancements in culture independent methods that can provide results more quickly and at a lower cost than culture dependent methods, and that employ genetic markers that focus on actual pathogenic strains, may increase the utility of surveillance as a means of triggering management actions.

CHAPTER III.

ENVIRONMENTAL INVESTIGATIONS OF *Vibrio parahaemolyticus* ILLNESS OCCURRENCE IN MASSACHUSETTS, 2014-2016

INTRODUCTION

Vibrio parahaemolyticus (Vp.) is a halophilic, Gram-negative, flagellate, rod-shaped bacterium and the leading cause of bacterial derived seafood poisoning in the US and worldwide (12-13). Human gastric infections from exposure to pathogenic strains of Vp. generally result in self-limiting gastroenteritis, headache, and fever. In rare cases, gastric Vp. infections can lead to life threatening septicemia; with the majority of severe Vp. infections observed in patients with compromised immune function (1, 14, 17). Vp. is naturally occurring and widely disseminated in coastal and estuarine waters globally; although; the vast majority of environmental Vp. strains are not known human pathogens and the baseline composition and abundance of pathogenic strains varies between areas (12-13). Environmental Vp. abundance generally demonstrates a seasonal trend, with the highest levels observed in summer months (25). In temperate latitudes Vp. becomes undetectable when water temperatures fall below 10°C (5, 25, 27), with levels rapidly increasing exponentially when water temperatures exceed 21°C (53). Vp. cells commonly attach to particulates and other organisms in the marine environment and can be found on a number of seafood products at market. Fully cooking seafood significantly reduces Vp. infection risk to consumers (1). As a result of consumer trends favoring raw consumption, and their ability to concentrate Vp. through normal filter feeding processes, oysters are the most commonly reported vector in seafood related Vp. infections with source information (1, 3-6).

In contrast to recent trends for other foodborne pathogens in the U.S., which have decreased over the past decade, the CDC reports the number of seafood related Vp. cases between 2012 and 2017 increased over 50% when compared to reported cases between 2007 and 2011 (11, 22). There has also been an observed northward shift in the geographic range of reported U.S. Vp. infections over the last two decades (10, 18, 22, 25, 27, 28, 29, 30, 36, 80-83). Historically, the majority of U.S. cases were associated with wild oysters harvested from the warm waters of the Gulf of Mexico (1, 12), an increasing number of U.S. sporadic infections and outbreaks have been attributed to shellfish harvested from the U.S. Pacific Northwest (PNW), British Colombia, and even as far north as Alaska (37, 80, 83, 85). In the summer of 2004, 62 people became ill after consuming raw oysters harvested from Prince William Sound, expanding the northern most point from which a Vp. outbreak had been reported by over 1000km (83). Likewise, since 2012 there has been a significant increase in the incidence of Vp. cases linked to the consumption of oysters harvested from the U.S. Northeast (NE) (26, 28, 30).

A number of factors are believed to be contributing to this northward shift in Vp. case occurrence. In the NE, the increased incidence of infections has been linked to the introduction of highly virulent pathogenic Vp. strains, as well as increasing virulence of endemic Vp. strains (25, 27, 28, 30). For example, in 2012 a Sequence Type-36 (ST36) strain commonly associated with outbreaks on the Pacific coast was identified in the first major U.S. outbreak in over a decade associated with oysters harvested from Oyster Bay, NY, which sickened over 28 people in 9 states. In the following year, ST36 strains resulted in an even larger oyster related Vp. outbreak, which lead to 109 infections across 13 states in the region (25, 27, 28, 30, 36). Since 2013 infections associated with ST36 have decreased in most Northeast states, but ST36 continued to result in the majority of cases in a number of Massachusetts harvest areas between

2014 and 2017 (28, 31), suggesting ST36 strains have established environmental reservoirs within the region (26, 28). In addition, infections associated with a resident strain known as ST631 have also increased in occurrence in the Massachusetts (29). Together, the ST36 and ST631 strains were responsible for 85% of local source gastric infections in Massachusetts between 2011 and 2017 (28, 29, 70).

While increased rates of U.S. Vp. infections are concerning, they must be put in the context of the corresponding increase in U.S. oyster production targeted almost exclusively toward the raw half shell market. For example, between 2008 and 2018, single oyster production in Massachusetts alone increased from 11 million pieces to over 50 million pieces (31), essentially increasing the rate of consumer exposure fivefold. With similar trends of increasing production reported in other states within the region and across the country (86), the observed reported increase in U.S. Vp. case occurrence may not actually constitute an increase in the risk per serving to consumers above the FDA estimated acceptable baseline risk of $\leq$1 illness per 100,000 servings (1, 7). However, production nor illness occurrence is equally distributed across the U.S., and to accurately determine if trends in increasing illness occurrence are being driven by changes in environmental conditions, increasing consumer exposure to Vp. bacteria, under-reporting rates, or a combination of factors, a detailed evaluation of harvest area specific production data and illness source attribution information is needed (7).

The Interstate Shellfish Sanitation Conference (ISSC) has established requirements in its National Shellfish Sanitation Program (NSSP) Model Ordinance (MO) for State Shellfish Control Authorities (SCAs) related to the assessment and mitigation of Vp. risk associated with the consumption of raw molluscan shellfish. In order to determine if the implementation of control measures to reduce the probability of Vp. illness occurrence are warranted in areas under

their jurisdiction, the NSSP MO requires SCAs to annually conduct a risk assessment to evaluate if the risk of Vp. infection from the consumption of oysters is reasonably likely to occur. In areas where environmental conditions or historic Vp. illness occurrence demonstrate that the risk of Vp. infection may be present, the NSSP requires State Shellfish Control Authorities to implement a Vp. control plan (VCP). The goal of the VCP is to reduce the probability of occurrence of Vp. illness through the establishment of time temperature controls or other measures, such as post-harvest processing or proactive closures, during periods that have been historically associated with annual illnesses. The environmental factors SCA are required to evaluate in their annual risk assessment are derived from the 2005 FDA Quantitative Risk Assessment on the Public Health Impact of Pathogenic *Vibrio* parahaemolyticus In Raw Oysters (1, 7); which uses quantitative product pathway analysis in which a number of factors that can influence the abundance of Vp. in raw oysters from harvest to consumption are evaluated, in part, to estimate differential Vp. risk between areas, harvest methods, and seasons.

The most influential factor affecting predicted risk of Vp. illness in the FDA assessment, and many other abundance based Vp. risk prediction models, is the level of total Vp. in oysters at the time of harvest, with water temperature as the primary environmental driver of Vp. abundance (1). However, Vp. population dynamics in coastal waters, and within the oyster tissue matrix, can be influenced by a number of complex factors associated with specific ecosystem conditions, overall climate conditions, short and long term fluctuation of conditions, and the effects and frequency of climatic events (18, 25, 27, 84). These factors can drive competition, growth, and evolution within ecosystems and individual Vp. communities, and within individual oysters, which can result in variability at the local and regional scale, and limit the utility of baseline regional risk assessment models, especially in temperate regions where there can be

significant variability in environmental conditions and Vp. population composition at a limited spatial extent (25, 27, 28, 37). To that end, numerous studies have attempted to identify the environmental determinates that correlate with Vp. abundance in U.S. harvest areas and apply those values to develop regional and localized risk assessment models (25, 27). A vast array of environmental parameters, including: temperature, salinity, chlorophyll *a*, turbidity, suspended sediments, nutrients, and dissolved organic carbon, have been demonstrated to have varying levels of influence on total Vp. abundance in shellfish at harvest (2, 5, 18, 25, 27, 42, 50- 51). However, the correlation of individual or multiple environmental parameters to total Vp. appears to be strongly variable between studies and study sites (42). As a result of this high level of variability, the majority of Vp. risk prediction models rely on the limited number of factors that tend to demonstrate broader geographic applicability and higher correlation; such as, temperature, salinity, and often chlorophyll *a*, (1, 18, 25, 27, 42).

Despite recent advances in Vp. detection methods and statistical predictive models (25, 27, 70, 72), the use of these tools to inform management practices remains limited. This is primarily due to the considerable uncertainty regarding the relationship between Vp. infection risk and total and potentially pathogenic Vp. indicator gene abundance in environmental oyster samples (25, 28, 36, 70). Often reported illness patterns do not correlate with the periods of predicted or observed high Vp. abundance, suggesting missing correlates that are not fully accounted for. For instance, recurrent, seasonal cases have occurred in the Pacific Northwest, not during summer when Vp. is predicted and observed to be most abundant, but in early spring (79, 87). While we should not discount the utility of abundance based risk assessment models to characterize Vp. infection risk conditions, it is clear from epidemiological reporting that differences in Vp. strain virulence and community composition between areas within a given

region can drive patterns in illness occurrence independent of environmental conditions (28). Further, few of these models have had the benefit of detailed epidemiological reporting to evaluate how abundance based risk assessment methodology actually correlates with documented illness occurrence beyond a regional and seasonal level. This is primarily due to the complexity associated with successful Vp. reporting and source attribution (Figure 1). In particular, due to the self-limiting nature of gastric Vp. infections, the CDC estimates there are 1.1 reported cases for every 142.4 unreported cases (13). In addition, less than 20% of U.S. Vp. cases reported to the CDC between 2012 and 2015 contained source attribution data, and fewer than 20% of those reports provided single source attribution data (11). Even for the small percentage of cases where a presumptive harvest area and harvest date can be identified, corresponding data on environmental conditions, and in particular Vp. levels in shellfish may not be available (12).

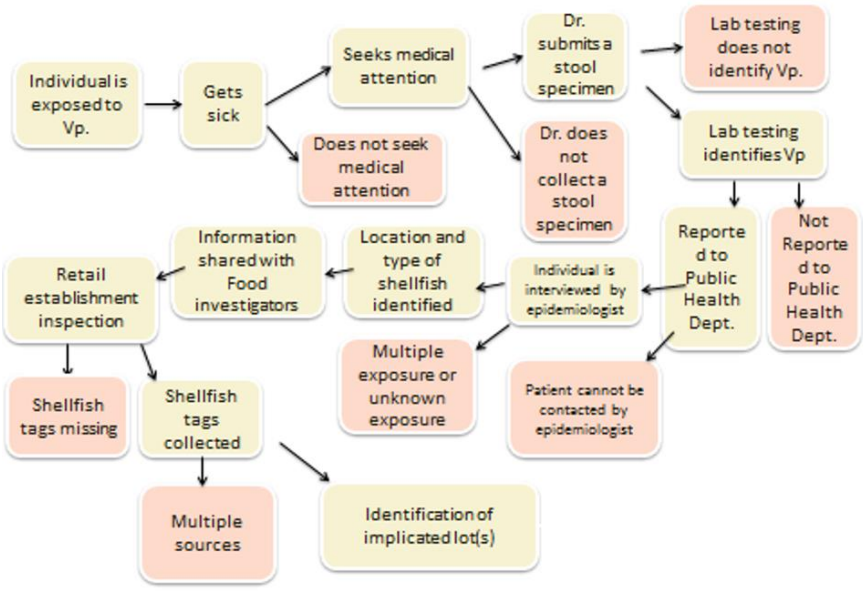


Figure 1. Vp. case trace back process. Light red boxes indicate a pathway that does not lead to the successful identification of source information.

Despite the limitations associated with Vp. illness source identification, between 2014 and 2016 Massachusetts Department of Public Health Epidemiologists and Seafood Inspectors were able to successfully identify the presumptive harvest area and harvest date for 67 laboratory confirmed gastric Vp. infections where Massachusetts oysters were identified as the presumptive vector (31). Of those 67 cases, 44 cases were linked to three distinct harvest regions in Massachusetts where information pertaining to the number and timing of Vp. cases, production levels, and environmental conditions in harvest areas on harvest dates implicated in cases was available. We analyzed this data in an attempt to determine how observed variability in environmental conditions within and between the three harvest areas influences Vp. infection risk in Massachusetts between 2014 and 2016. We also evaluated how our results compared with assumptions made in standard abundance based risk assessment methodology to determine the extent to which Vp. abundance modeling is a useful indicator of infection risk in Massachusetts. In addition, the sequence type of the clinical isolates in 39 of the 44 cases was evaluated to determine if there was variability in risk that may be associated with differences in Vp. communities between the studies areas. This analysis explores previously uninvestigated spatial and temporal interactions related to environmental conditions, Vp community composition, and illness occurrence; therefore, our work offers an original effort to evaluate how differences in Vp. community composition and environmental conditions can influence illness occurrence and Vp risk at a limited spatial and temporal extent, and how the use of epidemiological data can be applied to Vp. risk prevention and risk assessment methodology.

METHODS

Study Areas

The three study regions we evaluated (Figure 2) included: Katama Bay in the Town of Edgartown MA on Martha's Vineyard; Western Cape Cod Bay (WCCB), which includes harvest areas in Duxbury Bay in Duxbury, MA, and Plymouth Bay in Plymouth and Kingston MA; and Eastern Cape Cod Bay (ECCB), which includes Cape Cod Bay harvest areas in the Towns of Wellfleet, Barnstable, Dennis, Brewster, and Orleans, MA.

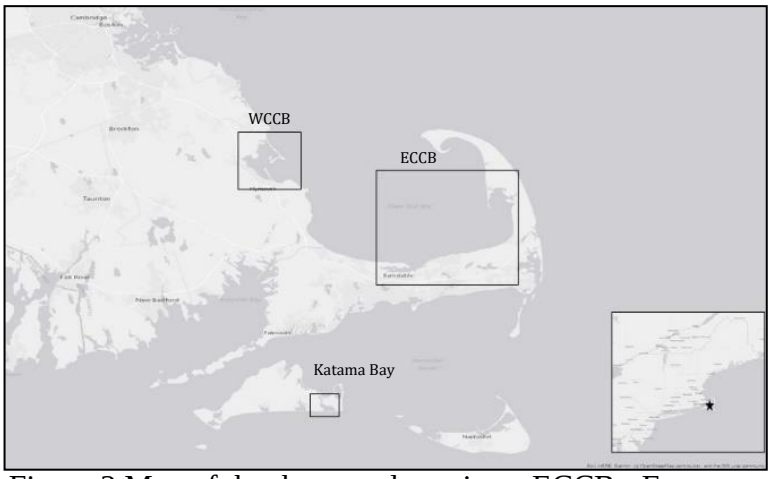


Figure 2 Map of the three study regions. ECCB= Eastern Cape Cod Bay, WCCB= Western Cape Cod Bay

These regions were defined based on hydrographic connectivity and relatedness of environmental conditions and harvest practices in the specific harvest areas contained in the individual harvest regions. All three regions are major oyster production areas and collectively represent subtidal tidal inlet systems (Katama Bay) and open bay intertidal (ECCB and WCCB) systems, as well as warm Gulf Stream influenced waters (Katama Bay), and cool Labrador Current influenced waters (ECCB and WCCB). This variability makes them ideal locations to evaluate differences in risk in the context of varying environmental conditions at a limited spatial

scale. All three harvest regions studied were under the same statewide Massachusetts Vibrio Control Plan between 2014 and 2016, which required icing within two-hours of harvest; however, specific icing times for individual implicated lots may have been less than two hours.

Illness source attribution data and harvest dates were obtained from public notices sent by the Massachusetts Division of Marine Fisheries (DMF), and from the Massachusetts Vibrio Program 2017 Vibrio Risk Assessment for Oysters (CITE). Data contained in notices and the risk assessment were derived from Massachusetts Department of Public Health foodborne illness investigations. All cases included in the analysis were laboratory confirmed as Vp. via genetic assessment of clinical isolates. Source attribution data are considered presumptive as attribution was obtained via oral patient interviews by DPH epidemiologists and wholesale and retail investigations conducted by DPH Seafood investigators. Records obtained at wholesale and retail establishments were confirmed with harvester records by DMF staff. For cases where a single harvest area was implicated, but multiple lots from different harvest dates were available for service on the date of consumption, environmental conditions from the harvest date most proximate to the date of consumption was used.

To determine the risk per serving for individual harvest regions, oyster production data were derived from dealer reported landings data. The quantity of oysters landed between May 15 and October 15 from the individual study regions were totaled, and values rounded down to the nearest thousandth for ease of reporting. To determine an appropriate value for the average percentage of oysters harvested in Massachusetts that were consumed raw during this period, we used a value provided in the 2017 Massachusetts Vp. Risk Assessment for Oysters of 95% (31). To develop a value for average serving size in Massachusetts, we used information reported on COVIS forms collected from patients by DPH epidemiologists during the foodborne illness

interviews for the cases included in this study; which include a question on the actual or estimated quantity of oysters consumed.

Environmental parameters, including surface (.5 meter) water temperature (°C) and salinity (ppt) were measured at 15-minute intervals from Duxbury Bay and Wellfleet Harbor and were used as representative data for WCCB and ECCB, respectively. Data were collected using YSI EXO2 multi-parameter data sondes (YSI, Inc. Yellow Springs, OH), owned and maintained by the Barnstable County Cooperative Program. In Katama Bay, water temperature (°C) and conductivity were measured via an Onset Computer HOBO UA-002-08 Temperature Pendant and U24-002-C Salinity Data Logger (Onset Computer Inc. Onset, MA), respectively. Upon retrieval, conductivity was converted to salinity with the HOBOware Conductivity Assistant (Version 2.1) that employs a non-linear temperature coefficient generated using the PSS-1978. Daily values for each parameter were averaged for analysis. Clinical isolates were sequenced via Illumina technology. Sequences of seven housekeeping loci were isolated from the resulting sequence data for each isolate to compare with allele combinations in previously reported strains in the MLST database.

RESULTS AND DISCUSSION

Our results and discussion are presented in the context of how our observations compared with assumptions in standard abundance based predictive risk assessment methodology currently employed in the U.S., and the utility of these observations to inform refine Vp. risk reduction management approaches for the Massachusetts.

The number and timing of* Vp. *cases linked to the consumption of oysters

During the study period harvest dates associated with Vp cases in Massachusetts occurred between June 15 and October 1 (Figure 6), with the peak period of illness occurrence between July 1 and September 15 (42/44 cases). Consistent with Vp. ecology in the region cases in the late fall, winter, and early spring were absent. The timing of overall and peak case occurrence was consistent with the seasonality of total and potentially pathogenic Vp. levels in Massachusetts harvest areas (Chapter 2; 5, 25).

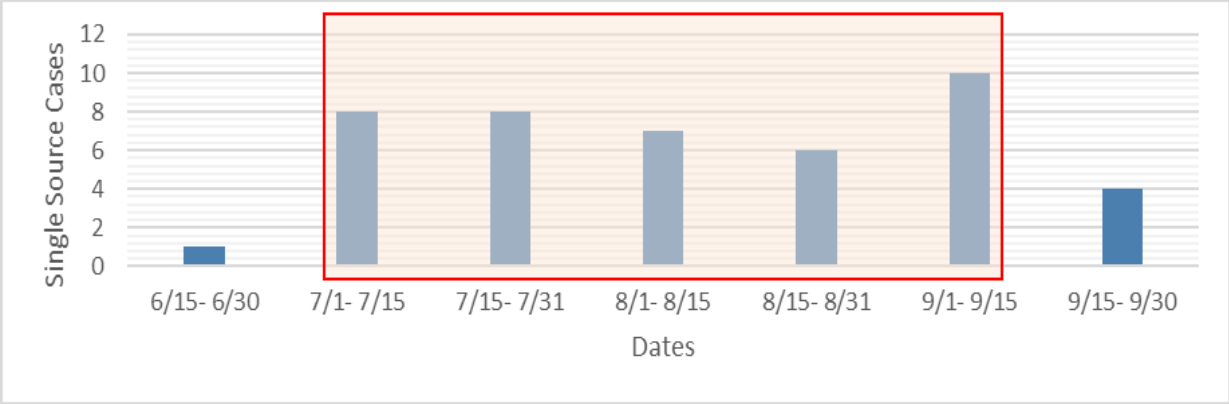


Figure 3 Epidemiology curve of single source oyster associated Vp. Illnesses attributed to three Massachusetts harvest areas (ECCB, WCCB, and Katama Bay) from 2014-2016. The red shaded box represents the observed "peak Vp risk period"

This suggests congruence with standard risk assessment methodology and provides a temporal basis for the establishment of a Vp. risk season in Massachusetts, during which the establishment of Vp. controls to limit risk of shellfish derived Vp. infections is prudent. The identification of a peak Vp. “risk period” also provides information for the period in which enhanced controls may be warranted in the state. While this provides a useful basis for statewide management, the number of cases and timing of peak case occurrence varied between study sites, with 17 cases (38%) linked to oysters harvested from Katama Bay, where the majority of cased

occurred during peak summer conditions, 16 cases (36%) linked to oysters harvested from WCCB, where cases were primarily associated with the latter half of the summer, and 11 cases (25%) linked to oysters harvested from ECCB, where illnesses were widely distributed across the entire Vp. risk season (Figures 4a-d). This suggests the initiation of elevated controls during the statewide peak risk may not correlate with harvest area specific trends and a more detailed evaluation of case occurrence in individual areas could inform a more nuanced approach.

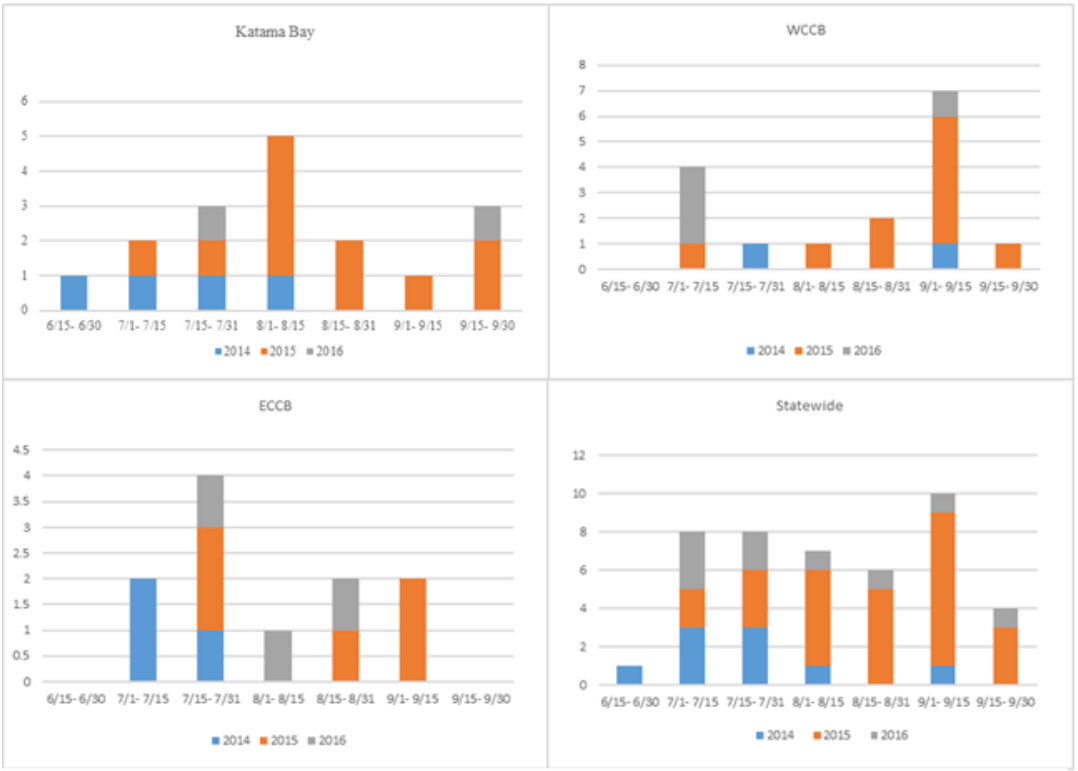


Figure 4. Epidemiology curves of sole source illnesses by year, date range, and implicated harvest region from 2014-2016

Environmental Conditions in Harvest Areas on Implicated Harvest Dates

An evaluation of temporal trends in Vp. illness occurrence may provide an indication of the general timing of Vp. risk in the state; however, environmental conditions can vary between years and within the risk periods, especially during shoulder seasons outside of the peak risk period, resulting in seemingly overly burdensome controls when conditions are unseasonably

cool, or inadequate controls during unseasonably warm conditions. Thus, in addition to establishing basic seasonal control measures on temporal trends in infections, a number of states have developed VCP that require the use of enhanced controls during periods when environmental conditions historically associated with the majority of infections are present. For example, in Connecticut harvest areas implicated in past outbreaks, all cases have been associated with water temperatures exceeding 20°C. This has allowed state managers to establish a 20°C water temperature threshold to define the peak risk period between years. When water temperatures exceed 20°C in those areas, the State notifies harvesters and implements enhanced post-harvest cooling measures (34). Washington State has also developed a VCP that employs environmental conditions as triggers for enhanced controls. Based on an evaluation of historic geographic and temporal trends in illness occurrence, state managers have divided the state into three risk categories. For each risk category they have established air and water temperature thresholds reflective of historic peak Vp. risk that correspond with the amount of time harvesters are required to place oysters under temperature control following harvest, with the highest tier outright prohibiting harvest until elevated risk conditions have abated. In Washington State harvesters are required to measure air and water temperatures prior to beginning harvest to determine the tier of cooling required on the date of harvest. This approach was employed as it provides greater flexibility to adjust to the spatially and temporally variable environmental conditions common to the PNW (37).

Whereas a number of environmental variables beyond water temperature can influence Vp. abundance in oysters (25, 27, 42), the vast majority of environmental thresholds in state VCPs are based water temperature, as it can be easily measured and thus functionally incorporated into a management scheme. To that end, we limited our analysis to water

temperature and salinity as such data was readily available and are the environmental variables most commonly reported to influence total and potentially pathogenic Vp. abundance in oysters from Massachusetts waters (Chapter 2, Table 1).

	Statewide	ECCB	Katama Bay	WCCB
Water Temperature (^{o}C)	23.8 (17.5-27.8)	24.6 (23.5-27.4)	24.9 (19.1-27.8)	19.9 (17.5-22.7)
Salinity (PPT)	30.3 (28.5-31.8)	30.5 (29.1-31.7)	29.8 (28.5-31.4)	30.5 (28.8-31.8)

Table 1. The range of environmental parameters reported on illness dates at the three study sites individually and combined.

Across all cases, the observed water temperatures in implicated harvest areas ranged from 17.5 to 27.8°C (median, 23.8°C) and salinity ranged from 28.5 to 31.8 ppt (median, 30.3 ppt). The narrow range of salinities present in harvest areas when illness were reported is characteristic of the majority of Massachusetts harvest areas, where trends in salinity are primarily influenced by tidal mixing and relatively stable (60). Due to this relatively narrow range, it is likely that salinity is not a valuable parameter for use to initiate enhanced control measures in Massachusetts. The wide range of water temperatures observed on implicated harvest dates also suggests challenges for the use of water temperature as a trigger to initiate elevated Vp. control measures on a statewide basis. While the 10°C range of water temperatures on infection dates is likely due to regional differences in water temperatures in Massachusetts driven by localized hydrographic conditions, and could potentially be accounted for through the development of localized control strategies such as those employed in Washington State, the

almost 9°C spread in water temperatures observed in Katama Bay on implicated harvest dates suggests even a localized approach would be challenging to employ in some harvest areas.

We evaluated the distribution frequency of environmental conditions on illness dates to identify if a narrower range of conditions was associated with the majority of cases. Table 2 describes the frequency distribution of environmental conditions on illness dates, statewide and for individual harvest areas. Statewide, approximately 75% of cases occurred when water temperatures exceeded 21°C and salinity was higher than 29 ppt.

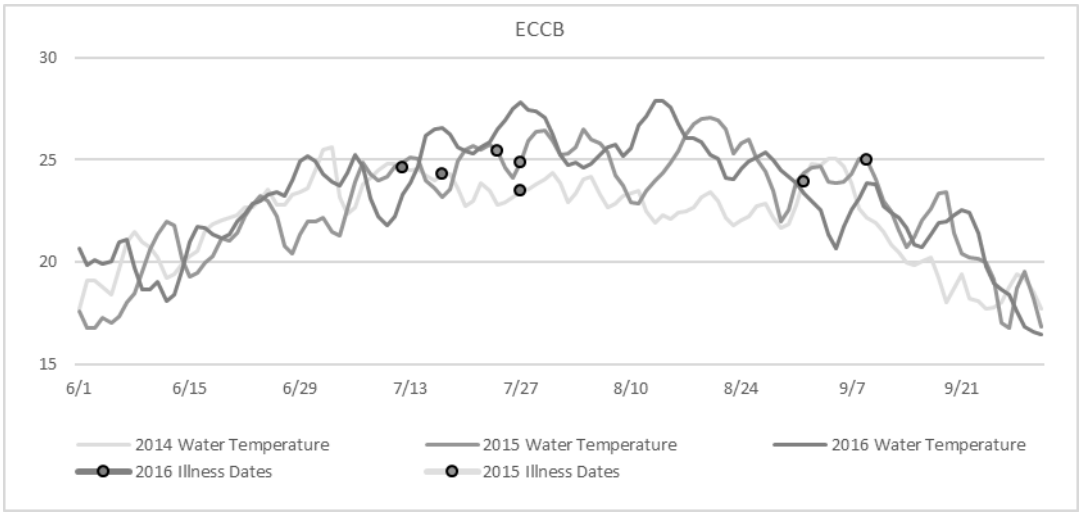
Average Water Temperature °C	Statewide	KB	WCCB	ECCB
27 To 28	9.10%	18.20%	-	9.10%
26 To 27	6.80%		-	18.20%
25 To 26	13.64%	36.40%	-	18.20%
24 To 25	18.20%	27.30%	-	45.50%
23 To 24	13.60%	18.20%	-	9.10%
21 To 22	9.10%	-	36.40%	-
20 To 21	4.50%	-	18.20%	-
19 To 20	19.20%	-	36.40%	-
17 To 18	2.30%	-	9.10%	-
Average of Salinity PPT	Statewide	KB	WCCB	ECCB
31 To 32	13.6%	5.9%	18.8%	18.2%
30 To 31	25.0%	23.5%	31.3%	18.2%
29 To 30	40.9%	52.9%	37.5%	27.3%
28 To 29	15.9%	17.6%	12.5%	18.2%
27 To 28	2.3%	-	-	9.1%
26 To 27	2.3%	-	-	9.1%

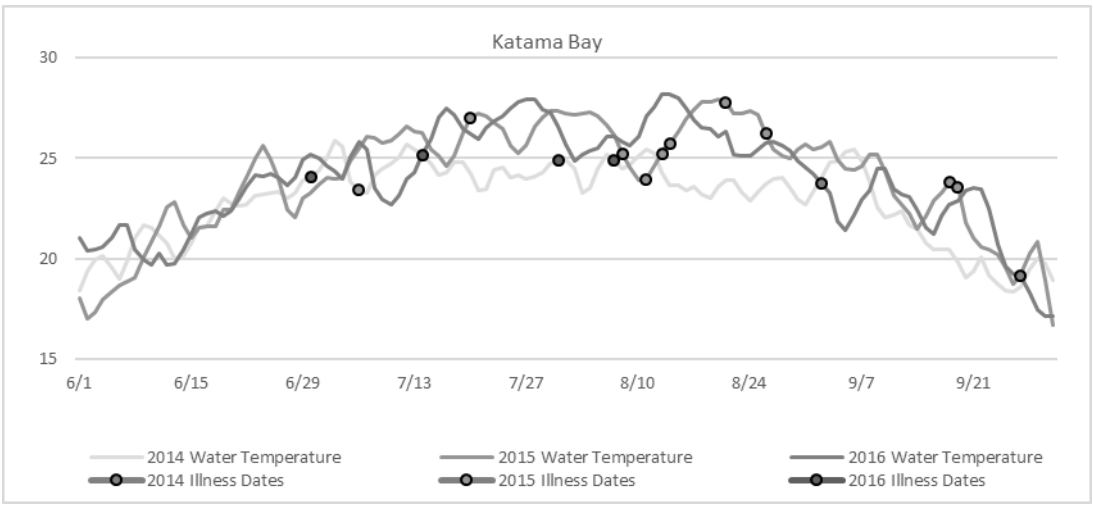
Table 2. Frequency distribution of environmental conditions on illness dates

Establishing triggers for elevated controls when these thresholds are exceeded could help to address the majority of the cases we evaluated, however, due to differences in the range of conditions associated with cases between harvest areas, they would fail to capture over 60% of cases attributed to WCCB and 20% of infections from ECCB. This analysis suggests that the use

of environmental triggers associated with infection occurrence would need to be based on area specific trends. However, it is important to note that the observed conditions on infection dates are frequently present in the three study areas, and other Massachusetts harvest areas, without corresponding reports of Vp. infections.

The observed interannual, seasonal, and short term variability in water temperatures in relation to Vp. infections demonstrates how the use of upper environmental thresholds for the initiation of elevated Vp. controls may fail to predict the periods of highest risk in some Massachusetts harvest areas (Figures 5a-c). In particular, despite a reported positive relationship between Vp. abundance and water temperature in all three harvest areas (Chapter 2), in many instances case occurrence did not correspond with peak water temperatures. For example, during an outbreak event in WCCB in 2015, six illnesses were linked to harvest dates spanning an 8 day period when water temperatures fell from 76°F to 68°F. In addition, in all three areas, the majority of cases (26/44, 59%) occurred in 2015, despite moderately significantly (P= 0.043) warmer temperatures at all sites in 2016.





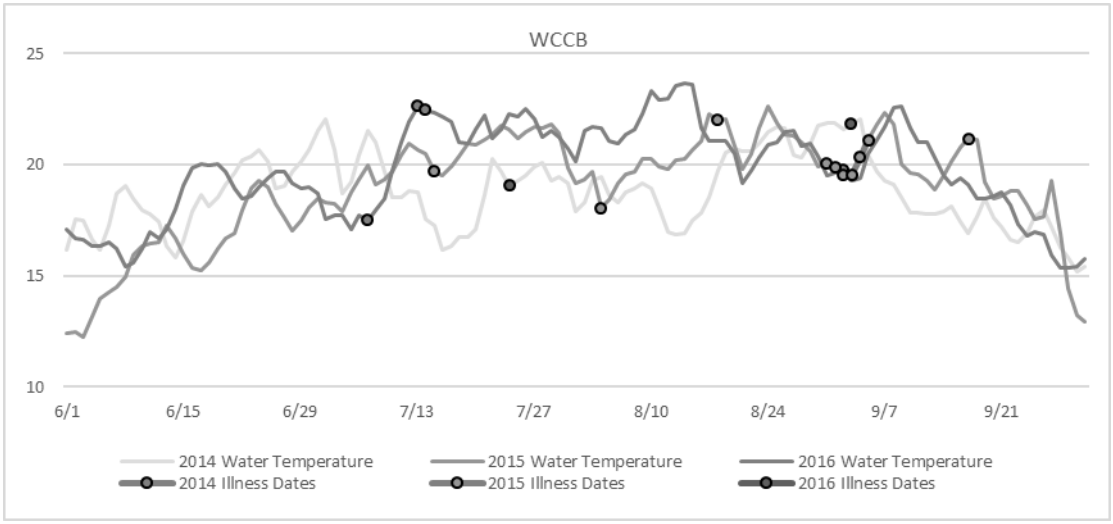


Figure 5 (a-c) Average daily water temperatures for each study site by year with dots representing illness dates.

Risk Per Serving

As the analysis of the environmental conditions on illness dates presented mixed trends in the association with case occurrence and environmental conditions commonly considered conducive to elevated Vp. risk within and between individual Massachusetts harvest regions, basing Vp. controls in the areas and during the periods the majority of infections have occurred will likely continue to serve as the basis for the state's approach to Vp. management. Based on the numbers of cases linked to the individual harvest regions it could be assumed that there is an approximately equal risk of Vp. infection in Katama Bay and WCCB, and that ECCB presents a substantially lower risk. Thus targeting enhanced controls in WCCB and Katama Bay would be appropriate. However, case numbers alone do not provide enough information to gauge how case occurrence actually corresponds with risk. In order to accurately evaluate how trends in case occurrence correspond with risk, case numbers must be evaluated in the context of oyster production between harvest areas and years. The FDA developed a Vp. risk calculator that allows managers to provide locally appropriate values related to production, serving size, and end use (% raw v. cooked) to calculate a risk per serving (RPS) (1). While this provides a tool to compare risk between areas with varying levels of production, there is little empirical data available to inform appropriate values for serving size and end use between regions. We employed a value for the average percentage of oysters harvested in Massachusetts that are consumed raw provided in the 2017 Massachusetts Vp. Risk Assessment for Oysters of 95% (31). This estimate is based on factors that indicate the dominance of oyster production directed at the raw half shell market, including: cage culture as the dominant method for oyster culture in Massachusetts, an average dockside price of oysters in the state between 2014-2016 of $0.57/piece, and a lack of wholesale facilities certified to shuck oysters (31). To develop a

regionally appropriate value for average serving size in Massachusetts we used information collected from patients by DPH epidemiologists during the foodborne illness interviews; which include a question on the actual or estimated quantity of oysters consumed. Though the accuracy of these numbers likely varies from case to case, and in some cases was not provided, values were available in 37 of the 44 cases and ranged from 1 to 24 oysters, with an average of 5.8 oysters per serving. This number is consistent with that reported by Walton et al. (2018) during a best worst choice survey identifying consumer preferences in trends in raw oyster consumption between the Northeast US and the Gulf of Mexico (88). When trends in illness occurrence are put in the context of production, we observed significant differences in the RPS between areas and years (Table 3). For example, while the number of cases associated with WCCB and KB during the study period were similar, due to the substantially higher production in WCCB, the risk in 2014 and 2015 was approximately 5 times higher in Katama Bay than in WCCB. This analysis highlights how case numbers when used alone can provide a misleading view of risk but also provides important context for further evaluation of factors that may lead to differential spatial and temporal Vp. risk in Massachusetts harvest areas. This is the first published estimate of Vp. RPS based on confirmed illness data and landings, and may serve as a model for other SCA to consider in their Vp risk evaluations.

Region	Year	~Production May 15-Oct 15 (pieces)	Cases	Total Servings (5.8 oysters/ serving)	Raw Servings (95% of production)	Cases/ 100,000 Servings
KB	2014	1,150,000	4	198,276	188,362	2.1
WCCB	2014	3,340,000	2	575,862	547,069	0.4
ECCB	2014	6,280,000	3	1,082,759	1,028,620	0.3
Combined	2014	10,760,000	9	1,855,172	1,762,413	0.5
KB	2015	1,160,000	11	200,000	190,000	5.8
WCCB	2015	5,230,000	10	901,724	856,638	1.2
ECCB	2015	6,790,000	5	1,170,689	1,112,155	0.5
Combined	2015	13,160,000	26	2,268,965	2,155,517	1.2
KB	2016	1,300,000	2	224,137	212,931	0.9
WCCB	2016	4,810,000	4	829,310	787,845	0.5
ECCB	2016	7,830,000	3	1,350,000	1,282,500	0.3
Combined	2016	13,920,000	9	2,400,000	2,280,000	0.4

Table 3. Calculation of the risk per serving for individual study areas from 2014-2016. KB= Katama Bay

Differential Risk Between Harvest Areas

Beyond water quality parameters, there are a number of factors that have been identified as having the potential to influence Vp. risk, such as culture and harvest practices, harvest site characteristics, observed Vp. levels, and pathogenic strain composition (1, 28). We evaluated how these factors may have influenced trends in Vp. infection risk between our study areas. The principal driver for estimated differential risk between culture and harvest practices is primarily associated with expected differences in the duration of post-harvest handling required to sort, count, bag, and tag oysters before they are placed under temperature control. For example, in WCCB many oysters are bottom planted and harvested from the seafloor via a collection basket. As this method generally precludes the sorting of oysters before harvest, and the basket does not exclude detritus and other materials, this method may require more sorting, and cleaning before the oysters can be placed under temperature control, than is required in areas where oysters are

harvested directly from cages, like in Katama Bay and ECCB. However, because between 2014 and 2016 all harvesters in Massachusetts were required to meet the same time to temperature requirements, regardless of harvest or culture method, we do not expect post-harvest handling due to the observed variability in harvest methods to have resulted in a significant portion of the observed variability in risk between sites. With that said, a number of recent studies have suggested that oysters harvested from floating or raised containers may have lower Vp. levels than oysters harvested directly off the bottom, likely due to decreased exposure to sediments and particulates that can serve as a reservoir for Vp. bacteria and be taken up during normal filter feeding activities (89). However, if this was a dominant factor influencing risk during our study we would have expected to see a lower RPS in Katama Bay, where oysters are cage cultured, than in WCCB where bottom harvest is the predominate harvest method.

Another factor that could drive variability in both baseline and intermittent risk between harvest regions in Massachusetts is tidal dynamics. Oysters exposed to elevated ambient air temperatures and radiant heating during low tide cycles are presumed to have a higher Vp. infection risk than oysters harvested from subtidal areas as a result of the potential for Vp. to accumulate in the oyster matrix during exposure (1). Due to the unique bathymetry in Massachusetts coastal areas, the extent oysters may be exposed to air during low tides can vary based on the location of the particular harvest site between the mean lower low water line and mean high water line, as well as astronomical and meteorological factors observed during the specific tidal period (60). South of Cape Cod Bay the tidal range is significantly smaller than that observed in most Cape Cod Bay harvest areas (31). For example, the tidal range in Katama Bay is less than 1 meter and all culture gear is located 1-1.5 meters below the mean lower low water line, resulting in no tidal exposure of oysters. Due to the long sloping shelf in ECCB, most

harvest sites are located well above the mean low water line, and oysters are routinely exposed at low tide. Harvest sites in WCCB are generally located below the mean low water line. As a result of the observed variability in tidal exposure between our study regions we would predict the highest risk in ECCB. In contrast, we observed the highest risk associated with oysters harvested from Katama Bay, and the lowest risk in ECCB. Further, as the increase in the RPS in 2015 was observed at all three study areas, regardless of tidal exposure, it is unlikely that tidal exposure was the driver of this intermittent increase in risk.

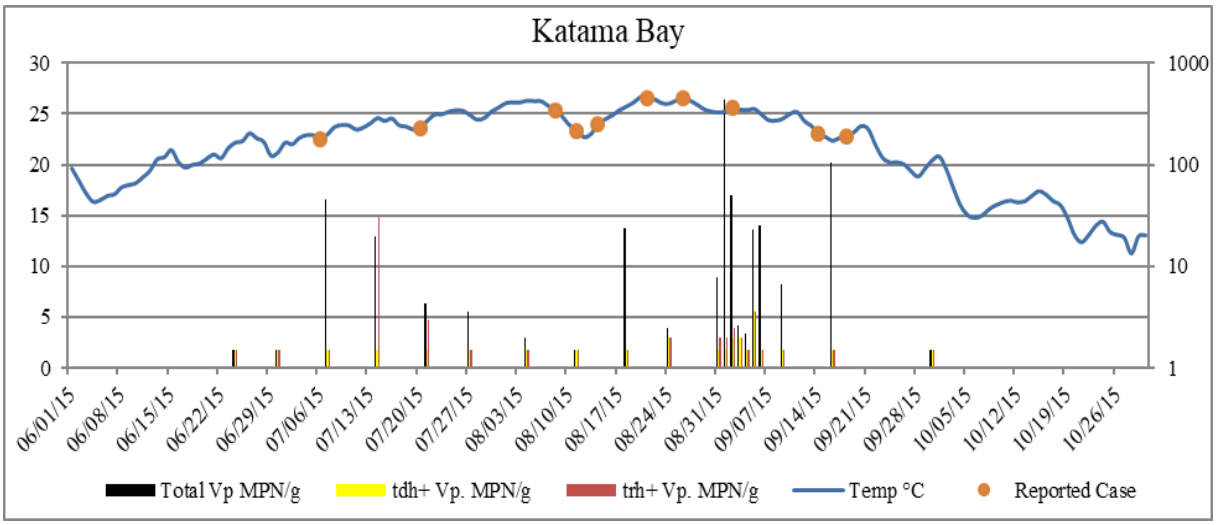
In the majority of abundance based Vp. risk assessment methodology water temperature is the most influential factor associated with Vp. risk. This is due to the correlation between increasing temperatures and increasing Vp. growth (1). As a result, we would estimate the highest risk in Katama Bay, followed by ECCB, and then WCCB. Our observations for Katama Bay, which on average had the highest observed water temperatures and highest risk RPS during the study period, were consistent with this assumption; however, observed average weekly water temperatures in ECCB during the study period were not significantly different than those observed in Katama Bay, but the observed risk in ECCB over the study period was on average 12 times lower than Katama Bay. Likewise, despite observed water temperatures in WCCB during the high risk period on average ~5 °C lower than ECCB, we observed a consistently higher RPS in WCCB than in ECCB; where we would expect to observe the lowest risk.

Vp. Levels in Proximity to Cases

As the majority of these factors are associated with Vp. risk due to their potential to influence abundance of Vp. in oysters, analysis of surveillance data capturing Vp. levels in oysters (1, 25, 27). In Chapter 2, we evaluated total and potentially pathogenic (*tdh*+ and *trh*+) Vp. levels in harvest areas located within our three harvest regions. A number of our findings

were consistent with our observations related to differential risk in the three harvest regions. For example, the average risk per serving between 2014 and 2016 in our three study areas were 2.9 cases/100,000 oysters in Katama Bay, 0.7 cases/100,000 oysters in Western Cape Cod Bay, where Duxbury Bay is located, and 0.37 cases/100,000 oysters in ECCB, where Wellfleet Harbor is located. Similarly, the maximum recorded value for *tdh*+ Vp. in Katama Bay was 2.97 log10MPN/g, which is roughly an order of magnitude higher than the maximum recorded value for *tdh*+ Vp. in Duxbury Bay of 1.63 log10MPN/g, and two orders of magnitude higher than the maximum recorded value for *tdh*+ Vp. in Wellfleet Harbor of 0.57 log10MPN/g. In addition, we also observed higher rates of detection and median levels of potentially pathogenic Vp., and higher relative percentages of *tdh*+ and *trh*+ Vp. to total Vp., in Katama Bay and Duxbury Bay, which are both located in areas where a higher infection risk was observed. This indicates there may be connection between periods of elevated total and potentially pathogenic Vp. in harvest areas and Vp. risk. While our sampling rarely overlapped exactly with case occurrence, we did collect Vp. surveillance data within our study areas proximate to reported infections. Figures 6a-b describe total and potentially pathogenic Vp. levels with trends in case occurrence for WCCB and Katama Bay in 2015. We observed elevated Vp. levels during periods in proximity to infection dates, however, we also observed cases in proximity to periods when total and potentially pathogenic Vp. were at or just above the limit of detection (Figure 6(a-b)). As we reported in Chapter 2, we also observed significant variability in Vp. levels from oyster samples at relatively limited spatial and temporal extents, and significant variability in Vp. levels from duplicate samples collected from the same harvest areas. Considering the observed variability in Vp. abundance within harvest areas over very limited time frames, it is likely that that current Vp. diagnostic methods and the frequency and scale of our surveillance efforts were not adequate

to determine if Vp. abundance from samples collected in proximity to Vp. cases are representative of Vp. abundance in the lots implicated in infections. It is also likely that adjusting controls to match trends in Vp. levels within harvest areas would generally be incompatible with the legal processes required to implement and enforce regulatory controls, and the practical aspects of notifying harvesters and enforcement officers that new controls are in place.



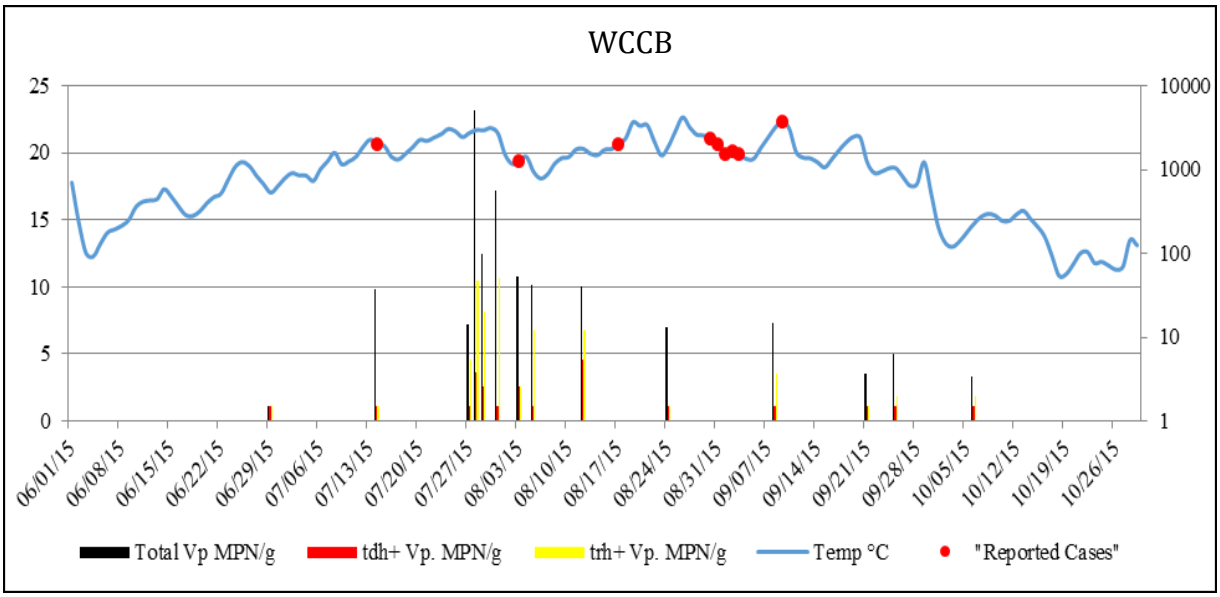


Figure 6(a-b). Total and potentially pathogenic Vp. levels with trends in case occurrence for WCCB and Katama Bay in 2015

Analysis of Clinical Isolates

One potential explanation for differences in risk between harvest areas is variability in the composition of pathogenic strain between our study areas. Isolates associated with Vp. cases attributed to Katama Bay were exclusively identified as ST36 with the exception of one isolate identified as ST 199, a broadly distributed Atlantic strain. Clinical isolates attributed to WCCB cases included ST36 (8/14) and ST631 (6/14). ST36 was associated in a half (4/8) of the illnesses from ECCB harvest areas, with the remaining isolates available for typing attributed to various endemic strain types with Atlantic lineages (4/8). Table 5 provides an overview of strain types associated with sole source illness by location from 2014- 2016 from each site.

Area	ST36	ST631	ST199	ST670	ST1156	ST1728	Not Available for typing
Katama Bay	16		1				
WCCB	8	6					3
ECCB	4		1	1	1	1	2

Table 4. Sequence types of clinical isolates from study areas (2014-2016). Three isolates from ECCB and 2 from WCCB were not available for typing.

Recent insights into pathogenic strain emergence in the Northeast suggest that differences in bacterial populations between harvest areas may play a larger role in enhanced disease risk than environmental conditions traditionally considered conducive for rapid Vp. growth (28,26). This hypothesis does fit a number of observed trends associated with differences in infection risk between areas. For example the observation of the highest RPS in Katama Bay, where almost all infections were attributed to ST36, is not surprising given the highly virulent nature of ST36 variants (Means). The strains associated with the ST36 complex appear to have a significantly smaller infectious dose than other pathogenic Vp strains and a wide thermal tolerance (18, 28,

37). Due to the lack of endemic strains implicated in infections traced to Katama Bay, it appears ST36 has dominated the pathogenic Vp. community, with the substantially higher risk per serving in Katama Bay almost certainly the result of the increased virulence of this strain. While ST36 was implicated in approximately half of the cases in WCCB and ECCB, endemic strains appear to be more prevalent north of Cape Cod Bay. In ECCB, where we observed the greatest diversity in the pathogenic strains associated with infections, we observed the lowest risk. This suggests greater competition between ST36 and endemic strains, potentially resulting in a lower abundance of this highly virulent pathogen as compared to endemic strains that have not been previously implicated in outbreaks and only appear to result in sporadic infections. While strains associated with infections in WCCB were a mix of ST36 and the endemic ST631, the ST631 strain has a genetically similar pathogenicity island as ST36, which may imply greater virulence (29, 73). The higher infection risk in WCCB over ECCB, is likely due in part to the larger proportion of the Vp. community in the area consisting of two strains with potentially enhanced virulence. However, WCCB demonstrated substantially lower infection risk than Katama Bay, suggesting that there may be differences in virulence between populations of ST36 that result in different levels of infection risk. Thus, additional knowledge about the genetic differences between the communities could be very informative for understanding differences in Vp. infection risk.

CONCLUSIONS

In summary, the current study evaluated the relationship between Vp. infection risk and various parameters in Massachusetts harvest areas. Our analysis identified clear seasonal and spatial trends in Vp. illness occurrence that can provide useful information for refining risk

management strategies in the State. For instance, the overall areas and timing where and when cases were reported were widely distributed across the risk period, supporting the general timing and extent of the current Massachusetts Vibrio control plan which is in effect statewide from approximately May 15- October 15. However, 75% of cases attributed to two areas, and 42 of the total 44 cases, occurred from July 1 and September 15, supporting enhanced controls in the two regions where the majority of illnesses were reported (WCCB, Katama Bay), and for the period of peak illness occurrence (July 1 and September 15).

This study reports the first accounting of relative infection risk based on state landings data of oysters. RPS varied between seasons, years and distinct hydrographic areas in MA. This was made possible by the availability of production data and detailed case investigation reporting. In 2015 the ISSC mandated States provide production data by month for all shellfish species, but was only able to require statewide values due to a lack of harvest areas specific reporting requirements in many states. This study highlights the value that would come from more refined reporting requirements, as well as a greater investment in case investigation.

Our study made clear the complexity of accounting for the effects of the introduction of a highly virulent non-native pathogenic Vp. strain with standard abundance based risk assessment methodology, and highlighted the value of the inclusion of clinical isolate analysis into risk assessment models. The growing use of culture independent diagnostic methods to diagnose Vp. infections may reduce the availability of clinical isolates for future efforts. Based on the value the isolate data brought to this study we recommend that, to the extent possible, states continue to require isolate submission and provide isolates for analysis.

LIST OF REFERENCES

1. USFDA. Draft risk assessment of the public health impact of *Vibrio parahaemolyticus* in raw molluscan shellfish. 2000.

2. DePaola A, Nordstrom JL, Bowers JC, Wells JG, Cook DW. 2003. Seasonal abundance of total and pathogenic *Vibrio parahaemolyticus* in Alabama oysters. Appl Environ Microbiol 69(3):1521-1526.

3. Givens CE, Bowers JC, DePaola A, Hollibaugh JT, Jones JL. 2014. Occurrence and distribution of Vibrio vulnificus and *Vibrio parahaemolyticus* – potential roles for fish, oyster, sediment, and water. Letters in Appl Microbiol 58(6):503-510.

4. DePaola A, Hopkins LH, Peeler JT, Wentz B, McPhearson RM. 1990. Incidence of *Vibrio parahaemolyticus* in U.S. coastal waters and oysters. Appl Environ Microbiol 56(8):2299- 2302.

5. Cox A, Gomez-Chiarri M. *Vibrio parahaemolyticus* in Rhode Island coastal ponds and the estuarine environment of Narragansett Bay. Appl Environ Microbiol. 2012;78(8):2996–9. doi: 10.1128/AEM.07519-11 (PMC free article) (PubMed)

6. Gooch, J. A., A. DePaola, J. Bowers, and D. L. Marshall. 2002. Growth and Survival of *Vibrio paramaemolyticus* in Postharvest American Oysters. Journal of Food Protection 6: 911-1053.

7. National Shellfish Sanitation Program (NSSP). 2017 Revision. Interstate Shellfish Sanitation Conference and the US Food and Drug Adminstration. http://www.fda.gov/Food/GuidanceRegulation/FederalStateFoodPrograms/ucm2006754.htm

8. Chakraborty S, Nair GB, Shinoda S. 1997. Pathogenic vibrios in the natural aquatic environment. Rev Environ Health 12(2):63-80.

9. Analysis of Vibrio vulnificus from Market Oysters and Septicemia Cases for Virulence Markers. Angelo DePaola, Jessica L. Nordstrom, Anders Dalsgaard, Anita Forslund, James Oliver, Tonya Bates, Keri L. Bourdage, Paul A. Gulig Applied and Environmental Microbiology Jul 2003, 69 (7) 4006-4011; DOI: 10.1128/AEM.69.7.4006-4011.2003

10. Baker-Austin, C., Oliver, J. D., Alam, M., Ali, A., Waldor, M. K., Qadri, F., & Martinez-Urtaza, J. (2018). Vibrio spp. infections. Nature Reviews Disease Primers, 4, 8.

11. CDC. National Surveillance of Bacterial Foodborne Illnesses (Enteric Diseases): National Cholera and Vibrosis Surveillance.

12. Daniels NA, et al. 2000. *Vibrio parahaemolyticus* infections in the United States, 1973–1998. J. Infect. Dis. 181:1661–1666 (PubMed)

13. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PJ. 2011. Foodborne illness acquired in the United States – Major pathogens. Emerg Infect Dis 17(1):7-15

14. Fujino T, Okuno Y, Nakada D, Aoyama A, Fukai K, Murai K, Ueho T. 1953. On the bacteriological examination of "shirasu" food poisoning. Med J Osaka Univ 4:299-304.

15. Hidetoshi U, Irma NGR. 2006. Aquatic environment. In The biology of Vibrios (eds Thompson FL, Austin B, Swings J), pp. 175–189. Washington, DC: ASM Press. (Google Scholar) (Ref list)

16. Colwell RR. 2006. A global perspective. In The biology of Vibrios (eds Thompson FL, Austin B, Swings J), pp. 3–11. Washington, DC: American Society of Microbiology. (Google Scholar) (Ref list)

17. Epidemiology, pathogenesis, and prevention of foodborne *Vibrio parahaemolyticus* infections. *Yeung PS, Boor KJ Foodborne Pathog Dis. 2004 Summer; 1(2):74-88.* (PubMed) (Ref list)

18. Froelich BA, Noble RT. Vibrio bacteria in raw oysters: managing risks to human health. *Philos Trans R Soc Lond B Biol Sci*. 2016;371(1689):20150209. doi:10.1098/rstb.2015.0209

19. Hoashi K, Ogata K, Taniguchi H, Yamashita H, Tsuji K, Mizuguchi Y, Ohomo N. 1990. Pathogenesis of Vibrio parahaemolyticus: intraperitoneal and orogastric challenge experiments in mice. Microbiol Immun 34(4):355-366.

20. Sanyal SC, Sen PC. 1974. Human volunteer study on the pathogenicity of Vibrio parahaemolyticus, p227-230. In Fujino T, Sakaguchi G, Sakazaki R, Takeda Y (ed), International symposium on Vibrio parahaemolyticus. Saikon Publishing, Tokyo.

21. Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Sharpiro C, et al. Food-related illness and death in the United States. Emerging Infectious Diseases. 1999;5(5):607–625. (PMC free article) (PubMed) (Google Scholar)

22. *Vibrio parahaemolyticus* trends, 2007–2015 Erin K. Burdette, MPH Surveillance Epidemiologist Interstate Shellfish Sanitation Conference *Vibrio parahaemolyticus* Workshop September 6-7, 2017

23. East Coast Shellfish Growers Newsletter, March 2018.

24. Committee on a Framework for Assessing the Health, Environmental, and Social Effects of the Food System; Food and Nutrition Board; Board on Agriculture and Natural Resources; Institute of Medicine; National Research Council; Nesheim MC, Oria M, Yih PT, editors. A Framework for Assessing Effects of the Food System. Washington (DC): National Academies Press (US); 2015 Jun 17. 2, Overview of the U.S. Food System. Available from: https://www.ncbi.nlm.nih.gov/books/NBK305173/

25. Urquhart, E.A.; Jones, S.H.; Jong, W.Y.; Schuster, B.M.; Marcinkiewicz, A.L.; Whistler, C.A.; Cooper, V.S. Environmental conditions associated with elevated *Vibrio parahaemolyticus* concentrations in Great Bay Estuary, New Hampshire. PLoS ONE 2016, 11, e0155018. (CrossRef) (PubMed)

26. Dynamic changes in Inoviridae prophage during population diversification, succession and Atlantic 1 invasion of Pacific-native Vibrio parahaemolyticus. Jillian Means1,2, Ashley L. Marcinkiewicz1,3, Randi L. Foxall1,2, Feng Xu1,4, Jeffrey A. Hall1,5, Stephen H. 3 Jones1,2, Vaughn S. Cooper1,5, Cheryl A. Whistler1,2- Pending Submission.

27. Hartwick MA, Urquhart EA, Whistler CA, Cooper VS, Naumova EN, Jones SH. Forecasting Seasonal *Vibrio parahaemolyticus* Concentrations in New England Shellfish. *Int J Environ Res Public Health*. 2019;16(22):4341. Published 2019 Nov 7. doi:10.3390/ijerph16224341

28. Xu, F.; Ilyas, S.; Hall, J.A.; Jones, S.H.; Cooper, V.S.; Whistler, C.A. Genetic characterization of clinical and environmental *Vibrio parahaemolyticus* from the Northeast USA reveals emerging resident and non-indigenous pathogen lineages. Front. Microbiol. 2015, 6, 272.

29. Xu, F.; Gonzalez-Escalona, N.; Drees, K.P.; Sebra, R.P.; Cooper, V.S.; Jones, S.H.; Whistler, C.A. Parallel evolution of two clades of an Atlantic-endemic pathogenic lineage of *Vibrio parahaemolyticus* by independent acquisition of related pathogenicity islands. Appl. Environ. Microbiol. 2017, 83, e01168-17.

30. Newton, A.; Garrett, N.; Stroika, S.; Halpin, J.; Turnsek, M.; Mody, R. Notes from the field: Increase in *Vibrio parahaemolyticus* infections associated with consumption of Atlantic coast shellfish—2013. Morb. Mortal. Wkly. Rep. (MMWR) 2014, 63, 335–336.

31. Massachusetts Division of Marine Fisheries Vp. Risk Assessment for Oysters, 2018

32. Lydon KA, Farrell-Evans M, Jones JL. 2015. Evaluation of ice slurries as a control for postharvest growth of Vibrio species in oysters and potential for filth contamination. J Food Prot 78(7):1375-1379.

33. Jones JL, Lydon KA, Kinsey TP, et al. Effects of ambient exposure, refrigeration, and icing on Vibrio vulnificus and *Vibrio parahaemolyticus* abundances in oysters. *Int J Food Microbiol*. 2017;253:54-58. doi:10.1016/j.ijfoodmicro.2017.04.016

34. Connecticut *Vibrio parahaemolyticus* Update 2017, DeRosia-Banick K.

35. Jones J, Lüdeke C, Bowers J, DeRosia-Banick K, Carey D, Hastback W. Abundance of *Vibrio cholerae*, V. vulnificus, and *V. parahaemolyticus* in Oysters (*Crassostrea virginica*) and Clams (*Mercenaria mercenaria*) from Long Island Sound. Appl Environ Microbiol. 2014;80(24):7667–72.

36. Martinez-Urtaza J, Baker-Austin C, Jones JL, Newton AE, Gonzalez-Aviles GD, DePaola A. 2013. Spread of Pacific Northwest *Vibrio parahaemolyticus* strain. N Engl J Med 369:1573-1574.

37. Flynn A, Davis BJK, Atherly E, Olson G, Bowers JC, DePaola A and Curriero FC (2019) Associations of Environmental Conditions and *Vibrio parahaemolyticus* Genetic Markers in Washington State Pacific Oysters. *Front. Microbiol.* 10:2797. doi: 10.3389/fmicb.2019.02797

38. Jacobs J, Rhodes M, Brown C, Hood R, Leight A, Long W, et al. Predicting the distribution of *Vibrio vulnificus* in Chesapeake Bay. NOAA Tech Memo NOS NCCOS. 2010;16.

39. Parveen S, DaSilva L, DePaola A, Bowers J, White C, Munasinghe K, et al. Development and validation of a predictive model for the growth of *Vibrio parahaemolyticus* in post-harvest shellstock oysters. Int J Food Microbiol. 2013;161(1):1–6. doi: 10.1016/j.ijfoodmicro.2012.11.010 (PubMed)

40. Urquhart E, Zaitchik B, Guikema S, Haley B, Taviani E, Chen A, et al. Use of Environmental Paramaters to Model Pathogenic *Vibrios* in Chesapeake Bay. J Environ Informatics. 2015;26(1):1–13.

41. Hsieh J, Fries J, Noble R. Dynamics and predictive modelling of *Vibrio* spp. in the Neuse River Estuary, North Carolina, USA. Environ Microbiol. 2008;10(1):57–64. doi: 10.1111/j.1462-2920.2007.01429.x (PubMed)

42. Takemura A, Chien D, Polz M. Associations and dynamics of Vibrionaceae in the environment, from the genus to the population level. Front Microbiol. 2014; 5(38).

43. FDA. Bacteriological analytical manual. http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm2006949.htm. Accessed 9-Aug-20.

44. Nordstrom JL, Vickery MCL, Blackstone GM, Murray SL, DePaola A. 2007. Development of a multiplex real-time PCR assay with an internal amplification control for the detection of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters. Appl Enviro Microbiol 73(18):5840-5847.

45. Panicker, G., D.R. Call, M.J.Krug, A.K. Bej. 2004. Detection of Pathogenic *Vibrio* spp. in shellfish by using multipliex PCR and DNA microarrays. Appl. Environ. Microbiol. 70:7436-7444.

46. Paranjpye R, Hamel OS, Stojanovski A, Liermann M. 2012. Genetic diversity of clinical and environmental *Vibrio parahaemolyticus* strains from the Pacific Northwest. Appl Environ Microbiol. 78(24):8631-8.

47. Honda, T., and T. Iida. 1993. *The pathogenicity of Vibrio parahaemolyticus and the role of thermostable direct hemolysin and related hemolysins.* Rev. Med. Microbiol. 4:106-113.

48. Hiyoshi, H., T. Kodama, T. Iida, and T. Honda. 2010. Contribution of *Vibrio parahaemolyticus* virulence factors to cytotoxicity, enterotoxicity, and lethality in mice. Infect. Immun. 78:1772-1780.

49. Deepanjali A, Kumar HS, Karunasagar I, Karunasagar I. 2005. Seasonal variation in abundance of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters along the southwest coast of India. Appl Enviro Microbiol 71(7):3575-3580.

50. Duan J, Su Y. 2005. Occurrence of *Vibrio parahaemolyticus* in two Oregon oyster-growing bays. J Food Sci 70(1):M58-M63.

51. Martinez-Urtaza J, Lozano-Leon A, Varela-Pet J, Trinanes J, Pazos Y, GarciaMartin O. 2008. Environmental determinants of the occurrence and distribution of *Vibrio parahaemolyticus* in the rias of Galicia, Spain. Appl Environ Microbiol 74(1):265-274.

52. Sobrinho P de S, Destro MT, Franco BDGM, Landgraf M. 2010. Correlation between environmental factors and prevalence of *Vibrio parahaemolyticus* in oysters harvested in the southern coastal area of Sao Paulo State, Brazil. Appl Environ Microbiol 76(4):1290-1293.

53. Falcioni T, Papa S, Campana R, Manti A, Battistelli M, Baffone W. 2008. State transitions of *Vibrio parahaemolyticus* VBNC cells evaluated by flow cytometry. Cytometry B Clin Cytom 74(5):272-281.

54. Kaysner C, DePaola A. *Vibrio cholerae*, *V parahaemolyticus*, *V vulnificus*, and Other *Vibrio* spp. Bacteriological Analytical Manual. 2004.

55. Randa, M. A., Polz, M. F., & Lim, E. 2004. Effects of Temperature and Salinity (PPT) on *Vibrio vulnificus* Population Dynamics as Assessed by Quantitative PCR. Appl. Environ. Microbiol. 70: 5469-5476.

56. Schuster. B.M. 2010. Microbial interactions with oysters from the Great Bay Estuary: characterization of the endemic *Vibrio cholerae* and oyster metagenetics.

57. Zimmerman AM, DePaola A, Bowers JC, Krantz JA, Nordstrom JL, Johnson CN, Grimes DJ. 2007. Variability of total and pathogenic *Vibrio parahaemolyticus* densities in northern Gulf of Mexico water and oysters. Appl Environ Microbiol. 73(23):7589-96.

58. Johnson C, Flowers A, Noriea N III, Zimmerman A, Bowers J, DePaola A, et al. Relationships between environmental factors and pathogenic *Vibrios* in the Northern Gulf of Mexico. Appl Environ Microbiol. 2010;76(21):7076–84. doi: 10.1128/AEM.00697-10 (PMC free article) (PubMed)

59. Parveen S, Hettiarachchi K, Bowers J, Jones J, Tamplin M, McKay R, et al. Seasonal distribution of total and pathogenic *Vibrio parahaemolyticus* in Chesapeake Bay oysters and waters. Int J Food Microbiol. 2008;128(2):354–61. doi: 10.1016/j.ijfoodmicro.2008.09.019 (PubMed)

60. Massachusetts Coastal Zone Management, 2015

61. Ceccarelli, D.; Hasan, N.A.; Huq, A.; Colwell, R.R. Distribution and dynamics of epidemic and pandemic *Vibrio parahaemolyticus* virulence factors. Front. Cell. Infect. Microbiol. 2013, 3, 97.

62. López-Hernández, K.M.; Pardío-Sedas, V.T.; Lizárraga-Partida, L.; Williams, J.D.J.; Martínez-Herrera, D.; Flores-Primo, A.; Uscanga-Serrano, R.; Rendón-Castro, K. Environmental parameters influence on the dynamics of total and pathogenic *Vibrio parahaemolyticus* densities in Crassostrea virginica harvested from

63. Mexico's Gulf coast. Mar. Pollut. Bull. 2015, 91, 317–329. (CrossRef) (PubMed)

64. Bartley CH, Slanetz LW. Occurence of *Vibrio parahaemolyticus* in Estuarine Waters and Oysters of New Hamsphire. Appl Environ Microbiol. 1971; 21(5):965–6.

65. O'Neill K, Jones S, Grimes D. Seasonal incidence of Vibrio vulnificus in the Great Bay estuary of New Hampshire and Maine. Appl Environ Microbiol. 1992; 58(10):3257–62. PMID: 1444362

66. O'Neill K, Jones S, Grimes D. Incidence of Vibrio vulnificus in northern New England water and shellfish. FEMS Microbiol Lett. 1990; 72(1–2):163–7.

67. Jones S, Summer-Brason B. Incidence and detection of pathogenic Vibrio sp. in a northern New England estuary, USA. J Shellfish Res. 1998; 17:1665–9.

68. Jones S, Striplin M, Mahoney J, Cooper V, Whistler C. Incidence and abundance of pathogenic Vibrio species in the Great Bay Estuary, New Hampshire. Proceedings of the Seventh International Conference on Molluscan Shellfish Safety. 2010.

69. Earle P, Crisley F. Isolation and characterization of *Vibrio parahaemolyticus* from Cape Cod soft-shell clams (Mya arenaria). Appl Microbiol. 1975; 29(5):635–40. PMID: 1096817

70. Whistler C, Hall J, Xu F, Ilyas S, Siwakoti P, Cooper V, et al. Use of Whole-Genome Phylogeny and Comparisons for Development of a Multiplex PCR Assay To Identify Sequence Type 36 Vibrio parahaemolyticus. J Clin Microbiol. 2015; 53(6):1864–72. doi: 10.1128/JCM.00034-15 PMID: 25832299

71. Canadian Food Inspection Agency. Canadian Food Inspection Agency. 2015. Available: http://www. inspection.gc.ca/

72. Kinsey TP, Lydon KA, Bowers JC, Jones JL. Effects of Dry Storage and Resubmersion of Oysters on Total Vibrio vulnificus and Total and Pathogenic (*tdh*+/*trh*+) *Vibrio parahaemolyticus* Levels. *J Food Prot*. 2015;78(8):1574-1580. doi:10.4315/0362-028X.JFP-15-017

73. Martinez-Urtaza, J.; Blanco-Abad, V.; Rodriguez-Castro, A.; Ansede-Bermejo, J.; Miranda, A.; Rodriguez-Alvarez, M.X. Ecological determinants of the occurrence and dynamics of *Vibrio parahaemolyticus* in offshore areas. ISME J. 2012, 6, 994–1006.

74. Lipp, E. K., A. Huq, and R. R. Colwell. 2002. Effects of global climate on infectious disease: the cholera model. Clinical Microbiology Reviews 15:757-770.

75. Rhodes, Matt & Bhaskaran, Hemalatha & Jacobs, John. (2013). Short-Term Variation in the Abundance of Vibrio vulnificus and *Vibrio parahaemolyticus* in a Tidal Estuary. Journal of Marine Biology & Oceanography. 02. 10.4172/2324-8661.1000109.

76. Grodeska SM, Jones JL, Arias CR, Walton WC. Effects of Desiccation Practices of Cultured Atlantic Oysters (Crassostrea virginica) on Vibrio spp. in Portersville Bay, Alabama, USA. *J Food Prot*. 2017;80(8):1280-1287. doi:10.4315/0362-028X.JFP-16-297

77. Kaufman, G & Bej, Asim & Bowers, J & Depaola, Angelo. (2003). Oyster-to-Oyster Variability in Levels of Vibrio parahaemolyticus. Journal of food protection. 66. 125-9. 10.4315/0362-028X-66.1.125.

78. Johnson, C.N.; Bowers, J.C.; Gritt, K.J.; Molina, V.; Clostio, R.W.; Pei, S.; Laws, E.; Paranjpye, R.N.; Strom, M.S.; Chen, A.; et al. Ecology of *Vibrio parahaemolyticus* and Vibrio vulnificus in the coastal and estuarine waters of Louisiana, Maryland, Mississippi, and Washington (United States). Appl. Environ. Microbiol. 2012, 78, 7249–7257.

79. Paranjpye RN, Nilsson WB, Liermann M, Hilborn ED, George BJ, Li Q, et al. Environmental influences on the seasonal distribution of *Vibrio parahaemolyticus* in the Pacific Northwest of the USA. FEMS Microbiol Ecol. 2015. December;91(12). (PubMed)

80. Baker-Austin, C.; Trinanes, J.A.; Taylor, N.G.; Hartnell, R.; Siitonen, A.; Martinez-Urtaza, J. Emerging Vibrio risk at high latitudes in response to ocean warming. Nat. Clim. Chang. 2013, 3, 73.

81. Baker-Austin, C.; Trinanes, J.; Gonzalez-Escalona, N.; Martinez-Urtaza, J. Non-cholera vibrios: The microbial barometer of climate change. Trends Microbiol. 2017, 25, 76–84.

82. Jones, S.H. Microbial Pathogens and Biotoxins: State of the Gulf of Maine Report. Gulf of Maine Council on the Marine Environment. Available online: http://www.gulfofmaine.org/2/sogom-homepage/ (accessed on 9, Aug 2020).

83. McLaughlin, J.B.; DePaola, A.; Bopp, C.A.; Martinek, K.A.; Napolilli, N.P.; Allison, C.G.; Murray, S.L.; Thompson, E.C.; Bird, M.M.; Middaugh, J.P. Outbreak of *Vibrio parahaemolyticus* gastroenteritis associated with Alaskan oysters. N. Engl. J. Med. 2005, 353, 1463–1470.

84. Nilsson,W.B.; Paranjpye, R.N.; Hamel, O.S.; Hard, C.; Strom, M.S. *Vibrio parahaemolyticus* risk assessment in the Pacific Northwest: It's not what's in the water. FEMS Microbiol. Ecol. 2019, 95, fiz027.

85. CDC. 1998. Outbreak of *Vibrio parahaemolyticus* infections associated with eating raw oysters - Pacific Northwest, 1997. MMWR 47(22):457-462.

86. USDA 2018 Aquaculture Census- https://www.nass.usda.gov/AgCensus/index.php Accessed 9, Aug, 2020

87. Turner, J.W., R. N. Paranjpye, E. D. Landis, S. V. Biryukov, N. González-Escalona, W. B. Nilsson, M. S. Strom. 2013. Population Structure of Clinical and Environmental *Vibrio parahaemolyticus* from the Pacific Northwest Coast of the United States. PLoS One. 2013; 8(2): e55726.

88. Petrolia, D., Walton, W., & Yehouenou, L. (2017). Is There A Market For Branded Gulf Of Mexico Oysters? Journal of Agricultural and Applied Economics, 49(1), 45-65. doi:10.1017/aae.2016.30